ENVIRONMENTAL AND MANAGEMENT FACTORS INFLUENCING SHRUB WILLOW BIOMASS YIELD AND QUALITY

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Eric S. Fabio

August 2017

ENVIRONMENTAL AND MANAGEMENT FACTORS INFLUENCING SHRUB WILLOW BIOMASS YIELD AND QUALITY

Eric S. Fabio, Ph. D.

Cornell University 2017

The need for sustainable, renewable energy production is growing as the impacts of human induced climate change are realized. Biomass is an abundant source of fixed carbon that can be converted to useful energy, and dedicated biomass crops such as shrub willow (*Salix* spp.) are expected to supply substantial amounts of biomass in the future. Shrub willow is a relatively new crop, however, and the genetic resources for improving yield and other biomass traits are only beginning to be explored. The variation in response to environment and management conditions have not been well characterized, especially for newly developed cultivars. The objectives of this research were 1) to identify shrub willow cultivars that display improved yield and biomass quality across two yield trial networks using current statistical methods, 2) to explore the variation in biomass composition among diverse cultivars and quantify the genetic and environmental contributions to variance, and 3) to test for differences in growth and physiological responses to nutrient additions. Using the Additive Main Effects and Multiplicative Interactions (AMMI) analysis, triploid hybrid cultivars were identified as having superior yields over diploid and tetraploid cultivars, and patterns of specific adaptability were identified using the AMMI mega-environment analysis. Subsequent use of mixed model versions of the AMMI analysis and other stability analyses confirmed superior performance in triploids for both older

and recently developed cultivars from the US breeding program. Biomass samples from the above yield trial networks were analyzed using High-resolution Thermogravimetric Analysis (Hi-Res TGA) to determine variation in biomass composition. Ash and hemicellulose content were found to be largely under genetic control, whereas cellulose and lignin content and biomass yield were largely controlled by environmental factors. Desirable biomass quality traits were positively correlated with yield, meaning that cultivar selection based on yield may have positive impacts on energy conversion processes. A greenhouse fertilization study showed that the current suite of species and species hybrids that have been developed for biomass production exhibit widely varying responses to nutrient availability, which can be exploited to apply the most appropriate cultivars for a given biomass project. Future research should focus on expanding genotype-by-environment and gene-by-environment analyses more widely to include the vast genetic resources available and on developing nutrient recommendations to better support future shrub willow biomass producers.

BIOGRAPHICAL SKETCH

Eric Fabio was born in Sumter, SC to Paul and Phyllis Fabio. As a child Eric always loved exploring his surroundings and expanding the boundaries of those surroundings. Living with expansive southern mixed oak-hickory forest in his backyard, Eric developed a love for plants and obtained botanical and taxonomic training under the mentorship of Dr. Gillian Newberry at the University of South Carolina, Spartanburg (now Upstate). With that knowledge, Eric explored the forests of both east and west coasts through various seasonal jobs in National Parks. The desire to secure a more permanent position led Eric to the University of Kentucky where he earned a Master's degree in forest ecology under Dr. Mary Arthur. After following his heart to Syracuse, NY, Eric worked with shrub willow at SUNY ESF and was excited by the potential for applied research to help to solve future energy challenges. Not long after, Eric's wife, Jessi Lyons, gave birth to their wonder identical twin boys, Elliot and Noah. While at SUNY ESF, Eric also got the chance to interact with Dr. Larry Smart, who later offered him the opportunity to earn a PhD studying shrub willow at Cornell University.

This dissertation is dedicated to my wife, Jessi Lyons, who has experienced all of my good and all of my bad during our 14 years together, including nine of those years in marriage. I cannot tell you what it has meant to me to have you by my side, receiving your love and support through it all. You are strong and intelligent, thoughtful, kind and caring. It weighs heavy on my heart that as you go through this life, spreading knowledge, you encounter those that would doubt your capabilities based solely on the fact that you are a strong, proud woman. Thank you for giving us our two beautiful, amazing twin boys, Elliot and Noah. You are such a wonderful mother and I merely take your lead in their upbringing. I hope that this degree will allow us to live better and provide a better life for our family and our community.

ACKNOWLEDGMENTS

First of all I would like to thank Dr. Larry Smart for the opportunity to pursue this degree. He has been an excellent mentor, always making time for his students. I have an immense amount of respect for Dr. Smart, as few people possess a combination of wit and intuition, along with compassion and patience as his does. His generosity over the years from always finding funding to support my research, to all the lab lunches in downtown Geneva is greatly appreciated. I am also thankful for the suggestions, insights and encouragement I received from my committee members, Dr. Stephen Reiners and Dr. Timothy Setter. They both possess an enormous range of knowledge, and have shown nothing but patience when I exhibit my lack thereof.

I am grateful to Mark Scott and Matt Christiansen for help with harvesting yield trials in the coldest of winter days. Steve Gordner provided excellent equipment operation, knowledgeable and practice advice and was always patient with me. He endured a lot of early mornings, late evenings and long road trips. I am thankful for Curt Carter for being a great technician and an even better friend. He always listened to my woes without complaint. I am indebted to the “WOW” team, Lauren Carlson, Dawn Fishback, Jan Petzoldt and Rebecca Wilk. They processed countless samples of all sorts, endured long road trips and aching wrists and knees with no complaints. Thanks to Lauren for always laughing at my jokes, even when they weren’t funny. Thanks to Dawn for the careful attention to detail and the motherly love. Thanks to Jane organizing data and for making me smile. And thanks to Rebecca for managing it all.

I am grateful to my labmates, Fred Gouker, Michelle Serapiglia and Craig Carlson for letting me ask so many silly questions and vent my frustrations and for your excellent advice. I would also like to thank the many folks that helped in the field and the lab over the years,

including Michael Rosato, Cody Laflar, Nick Durnin, McKenzie Schessl, Lindsey Mattic and Brian DeGasperis. Thanks to Dennis Rak for hosting trials, applying treatments and giving good advice.

I cannot thank Hugh Gauch, Jr enough for teaching me about the AMMI model and the analysis of yield trials. He donated his time to help me learn the use of his software, MATMODEL, and to interpret the output. I wish him the best of luck in the release of his new program, AMMISOFT.

Funding for the early yield trials was provided by the North Central Regional Sun Grant Center at South Dakota State University through a grant from the US Department of Energy Bioenergy Technologies Office and the US DOE Regional Feedstock Partnership. The second round of cultivar testing was supported by a grant from USDA-NIFA through the Northeast Sun Grant Initiative and by Agriculture and Food Research Initiative Competitive Grant from the USDA NIFA for the NEWBio Coordinated Agricultural Project. The greenhouse experiment and the cover crop trials were funded through a USDA NIFA Federal Capacity Funds grant.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Effects of nitrogen fertilization in shrub willow short rotation coppice production-A review

1.1 Abstract

Sustained interest in producing renewable energy from willow (*Salix* spp.) short-rotation coppice (SRC) production has resulted in a substantial amount of published research over the past few decades. One area of consistent focus has been the nutritional requirements for optimal growth and yield of willow. Inconsistency in the results of these research efforts has perhaps been a driver of renewed experimentation. This review is intended to provide a qualitative and quantitative examination of the effect of fertilization treatments on willow biomass yield. Data from the literature was collected in order to summarize variation in important nitrogen (N) pools in willow biomass of common species used in SRC research programs in North America and Europe. Studies comparing willow fertilized with synthetic or organic sources of N to an unfertilized control treatment were analyzed for treatment effects. Overall the majority of responses to fertilization were positive, although a large amount of variation by species, treatment type and treatment level were found. Environmental and economic aspects are also considered.

1.2 Introduction

1.2.1 Background and focus

Production of dedicated bioenergy crops such as shrub willow is often said to be targeted for marginal land, because they require lower inputs than traditional commodity crops and thus could make marginal lands more profitable while avoiding conflicts with producing energy on land that should be used for food production. While the definition of marginal land can be vague

and is often not stated (Richards et al., 2014), limitations often include poorly or excessively drained soils, steep slopes, degraded lands or nutrient limitations.

When considering nutrient demand in shrub willow for biomass production, conflicting or contradictory statements can be found in the literature: “Nutrient demands of short-rotation willow plantations are high” (Simon et al., 1990); “Based on the nutrient budget results from the initial 4-year rotation, we found SRC willow to be relatively low nutrient-demanding, with minimal nutrient export from the plantations other than in harvested biomass” (Hangs et al., 2014b); “Fast growth makes the high yielding plants nutrient demanding, since growth and nutrient uptake are closely linked processes” (Ericsson, 1994). It could be argued that research into nutrient demand and fertilization response over at least 25 years has not produced any definitive conclusions on the appropriate amounts or timing for fertilizer applications, prompting dismay from authors that have sampled the literature: “Despite the extensive research on fertilizer use in SRC systems, the optimal time to apply fertilizer is still unknown for SRC systems in North America” (Amichev et al., 2014); “…the reported growth response of numerous willow varieties to added fertilizer N when grown under field conditions has been inconsistent, thereby precluding definitive relationships (i.e., calibrated fertilizer recommendations) between applied fertilizer N rates and subsequent willow biomass yields from being developed and applied universally” (Hangs et al., 2012); “Despite decades of research on fertilization of shrub willow, fertilizer requirements are still uncertain, and analysis of willow studies in the region showed no correlation between N-fertilizer rate and biomass yield” (Stoof et al., 2015).

In Scandinavia, arguably the region with the longest experience with management willow SRC for biomass production, there seems to be renewed interest in quantifying the response to

fertilization. Aronsson et al. (2014) argued that existing recommendations for fertilization of shrub willow in Sweden may be out of date on economic grounds, based on changes in prices of woody biomass and costs of fertilizer. They also argue that plant breeding has produced cultivars that have a more positive response to fertilization compared with older cultivars that were in production when earlier recommendations were formed. In neighboring Denmark, new studies have investigated fertilization response in modern commercial Swedish cultivars (Larsen et al., 2016; Sevel et al., 2014), and in reviewing previously published research on fertilization effects on yield, the authors deplore the lack of consensus among previous studies.

The quiet debate in the literature seems to be whether fertilization should only occur in order to replace nutrients removed at harvest, or if it should be used as a management tool to improve yields. The economics of the latter should be a major consideration (Aronsson et al., 2014), however it is rarely discussed. This point of contention becomes very important when considering the economic and environmental aspects of willow SRC production, since yield is widely perceived to be extremely important in determining profitability in SRC biomass production (Hauk et al., 2014). Furthermore, after harvesting operations, fertilization can be the largest energy input and the majority of that energy is consumed in the manufacturing of the fertilizer (Djomo et al., 2011). Not only is unutilized fertilizer economically and energetically wasteful, N and P lost through leaching or gaseous losses through denitrification and N_2O production can contribute to eutrophication in waterways and increased global warming potential. These aspects will be discussed in detail below, including a literature review of for quantifying these impacts.

1.2.2 Nitrogen requirements and fertilization recommendations

It is often stated that shrub willow stands can cycle nutrients efficiently, that leaves hold a

significant pool of many essential nutrients, and that harvesting should therefore occur after leaf fall so that nutrients contained within leaves are retained on site and can be cycled through decomposition (Ericsson, 1994). Therefore, harvesting during dormancy and after litterfall should reduce nutrient removal and thus lessen the need for subsequent amendments. Likewise, resorption of nutrients and translocation to the roots for storage in dormancy should also benefit nutrient retention, although pot studies have indicated that stems of shrub willow can be a significant sink for N during dormancy, which likely allows for more rapid growth in the spring at budbreak (Bollmark et al., 1999; Brereton et al., 2013). However, this characteristic also means that a significant portion of N (and other nutrients) can be removed at harvest, which has been a consideration and/or concern since the early days of research on shrub willow production for bioenergy in Europe (Ericsson et al., 1992; Mitchell et al., 1999).

Broad fertilization recommendations published over the years, formulated on an annual basis and across the first two rotations have dropped dramatically since the 1980's (Table 1.1). Aronsson et al. (2014) provided an excellent review of commercial willow production in Sweden and noted that early recommendations were developed using older cultivars that likely have low nutrient use efficiencies and lower resistance to pests and diseases compared to contemporary cultivars. Aronsson et al. (2014) also suggest that decreased valuation of biomass relative to management costs could have discouraged producers from fertilizing commercial fields in Sweden. They also speculate that some recommendations in other countries may be lower, because they are based on N removal from harvest, instead of maximizing growth. Indeed, numerous other studies elected to report N removal rates instead of explicitly making fertilization recommendations (Table 1.2).

Table 1.1 Published N fertilization recommendations.

Country	Study	Harvest cycle	Year in cycle	N (kg ha^{-1}) recommended	Cumulative within cycle
Sweden	Sennerby-Forsse (1986)*	1	1	80-110	
		1	2	60-80	
		1	3	60-80	200-270
		2	1	60-80	
		2	2	60-80	
		2	3	60-80	180-240
Sweden	Ledin et al. (1994)	1	1	45	
		1	2	100-150	
		1	3	90	135-285
		2	1	60	
		2	2	100	
		2	3	60	220
Sweden	Danfors et al. (1997)	1	1	45	
		1	2	100-150	
		1	3	0	145-195
		2	1	100-150	
		2	2	100	
		2	3	0	200-250
USA	Abrahamson et al. (2010)	1	1	100	100
		2	1	100	100
Denmark	Aronsson et al. (2014)	1	1	0	
		1	2	0	
		1	3	0	0
		2	1	60	
		2	2	100	
		2	3	60	220

*From Ericsson (1994)

Table 1.2 Published N removal rates at harvest intended to serve as recommendations for N replacement rates after harvest.

Country	Study	Rotation	N export (kg ha^{-1})	Yield (Mg ha^{-1} yr^{-1})
Sweden	Ericsson (1994)	1	128	13
		2	136	14
USA	Adegbidi et al. (2001)	1	75-86	15-22
Ireland	Caslin et al. (2010)	1	150-400	8-10
UK	AHDB (2017)	1	90	10

All of these estimates were derived from experimental situations, and a number of studies apply fertilization treatments during years of production when stem heights would preclude any practical mechanical means of applying fertilizer in a commercial setting, which was recognized

by some authorities (Abrahamson et al., 2010; Agriculture and Horticulture Development Board, 2017). These authorities also state that no fertilizer is required during the establishment year, and assume that the first year of growth is coppiced, followed by a three-year rotation, which may no longer be a universal practice (Albertsson et al., 2014). Recommendations from the US suggest that 100 kg N ha^{-1} can be applied in the second year of growth, after the establishment year growth has been coppiced, which stimulates rapid regrowth of multiple stems (Abrahamson et al., 2010). However, they advise against fertilizing if weeds are a problem at the start of the second year.

There are no specific recommendations given for the beginning of the second three-year rotation period, however Sleight et al. (2016) indicated that most US yield trials are fertilized with an additional 100 kg N ha^{-1} the spring following harvest of the first three-year rotation. Mitchell et al. (1999) suggest that if planted "on land previously used for arable cropping" there is no yield benefit to fertilizing in the first 10 years of production. Indeed, other sources have cautioned against fertilization in the first or second year of production because of poor uptake ability of young plants with undeveloped root systems (Aronsson et al., 2010), and because of the potential for weed competition (Albertsson et al., 2014). Aronsson et al. (2014) suggested updating Swedish fertilization recommendations suggesting no fertilizer be used at all during the first rotation. However, a majority of studies investigating the effects of fertilization on yield have involved treatments applied in the first rotation. Adegbidi et al. (2001) report on a number of fertilizer response studies in the US covering numerous species, fertilizer rates and timing of applications and consequently provide N removal rates. There were no explicit fertilization recommendations found for Canada, although Guidi et al. (2013) summarized nutrient removal rates in references from Sweden, the US and UK, which essentially match those summarized

here (Table 1.2).

Work on nutrient cycling in field grown, non-fertilized willow trials in Saskatchewan, SK allowed for the development of detailed nutrient input/output budgets for N and other macronutrients through the first three-year rotation (Amichev et al., 2014; Hangs et al., 2014b). These studies demonstrated that if foliage and belowground biomass are considered outputs, then there is a net loss of N after the first harvest, including N contained in the harvested stems. However, if these tissues are considered to be sinks that retain N through a continual process of turnover, then there was a significant surplus of N at the end of the rotation compared to the initial available N at planting. Leaching losses were determined to be minimal, and N deposition was three times greater. If establishment of willow stimulated soil organic matter turnover, and mineralized N was taken up and stored in litter and belowground biomass, the surplus scenario seems plausible. However, the question remains whether or not the harvesting of stems stimulated a reduction in belowground biomass (Berhongaray et al., 2015; Cerasuolo et al., 2016), which could result in N losses after harvest (Dimitriou et al., 2012). Hangs et al. (2014b) ultimately recommend a conservation replacement rate of 25 kg N ha^{-1} after each harvest to ensure adequate N supply for future growth.

The aim of this review is to examine the yield response to fertilization in SRC willow through both quantitative and qualitative means. Consideration is given to dominant biomass N pools and the effects of types and amounts of fertilization. Environmental and economic considerations are also examined.

1.3 Nitrogen pools in biomass and effects of fertilization on biomass production

1.3.1 Literature data sources

This review was focused only on N fertilization as most of literature has focused on N

applications. Although a number of studies have incorporated P and K additions into N fertilization treatment, few have quantified their effects (although see Hytönen, 1995). A literature search was conducted using ISI Web of Science and Google Scholar databases. Keywords '*Salix'*, or 'willow' in combination with 'fertilization', 'biomass', 'nitrogen'. Only studies published in English were considered. Resulting sources were reviewed for two types of observations; 1) foliar and/or stem tissue N concentrations in combination with yield observations, or 2) reports of yield from fertilization treatments with an unfertilized control treatment for comparison. Only field based studies were considered for both types of data. The majority of studies reported yields from stands that were harvested at 2 to 3 years of age, but some studies included in the final database reported stands at 1, 4 or 5 years of age at the time of harvest. Mean values were obtained from tables or if only figures were available, WebPlotDigitizer V 3.12 software (Rohatgi, 2017) was used to extract data values. Within each study, if multiple cultivars, multiple locations or cultivar-location combinations were tested, these were considered independent observations in the database. Studies conducted in the context of soil remediation were avoided due to confounding edaphic conditions that may affect growth and response to nutrient availability. In addition, observations were restricted to the most frequently occurring species or species hybrids. For the foliar and stem N concentration dataset, there were 13 separate studies published between 1998 and 2016, with a total of 82 observations. For studies reporting stem biomass N concentrations, the total amount of N removed at harvest (kg N ha^{-1}) was estimated using the annualized yield (Mg ha^{-1} yr^{-1}), the age of the stems harvested (yr) and the N concentration of the stems (kg N Mg^{-1}). The N use efficiency for each observation was calculated as the amount of biomass produced per unit N (kg biomass kg^{-1} N). For this group of observations, fertilization treatments were not considered, but instead the focus

was base level N requirements of crops. The fertilization treatment dataset contained 23 separate studies published between 1995 and 2016, with 171 independent observations. The percentage increase (or decrease) in annualized yield was calculated as $(Yield_{Fert} - Yield_{cont})/(Yield_{cont})*100$. Fertilizer use efficiency was calculated as the difference between fertilized and control treatment yields divided by the total amount of fertilizer applied.

An attempt was also made to gather data on soils properties for each study or locations within studies. The most often reported values were soil pH and % soil organic matter (SOM) or % soil C. Any reported values of SOM were converted to % C by multiplying by a factor of 0.58. These parameters were used as possible predictor variables.

1.3.2 Canopy N concentrations

During the growing season, leaves comprise the largest pool of plant N in order to support photosynthesis. Some studies have demonstrated that canopy N concentrations can be an indicator of stem dry matter biomass accumulation. For instance, Ens et al. (2013) demonstrated that foliar N concentration could predict about two thirds of the total variation in *S. purpurea* harvested biomass across seven contrasting locations in central Canada. In contrast, Jug et al. (1999) determined that the use of foliar N concentration as an indicator of biomass production of *S. viminalis* was site dependent and found relatively weak correlations. They also observed that only fertilization treatments allowed *S. viminalis* leaves to exceed 30 mg N g^{-1}, which also had the greatest yields. Labrecque and Teodorescu (2001) found that unfertilized *S. viminalis* grown in eastern Canada obtained foliar N concentrations above 30 mg N g^{-1} on two clay soil sites, but only reached 24 mg N g^{-1} on a sandy soil site. There was some support for this trend across the five species or species hybrids from nine studies found in the literature that had mid-growing season foliar N concentrations and end of season biomass yields (Figure 1.1). The exceptions

were *S. viminalis*, which seemed to have high foliar N, but relatively low harvested biomass, and *S. miyabeana* that displayed the opposite trend, although this species had a wide range in foliar N.

The distribution of N in the foliar canopy may also affect stem biomass production, not just the concentration. Weih and Ronnberg-Wastljung (2007) demonstrated that *S. viminalis* cultivars can have steep vertical N gradients, meaning that foliar N concentrations change rapidly from shade to sun leaves. In contrast, *S. dasyclados* cultivars have a more even vertical distribution of N through the canopy. Hybrids between *S. schwerinii* and *S. viminalis* had intermediate N gradients. However, it was the more evenly distributed canopy N species that showed the greatest shoot biomass.

Kopinga and Van den Burg (1995) used foliar nutrient analysis to assess the nutritional status of three willow species, including *S. viminalis*. They determined that below 18 mg g^{-1} was inadequate, between 22 and 30 was adequate and above 30 was optimal. Weih and Ronnberg-Wastljung (2007) described senescent *S. viminalis* leaves as having approximately 18 mg N g^{-1} or less, while Hangs et al. (2014a) measured between 9 and 14 mg N g^{-1} in senescent leaves of *S. miyabeana* and *S. purpurea* cultivars.

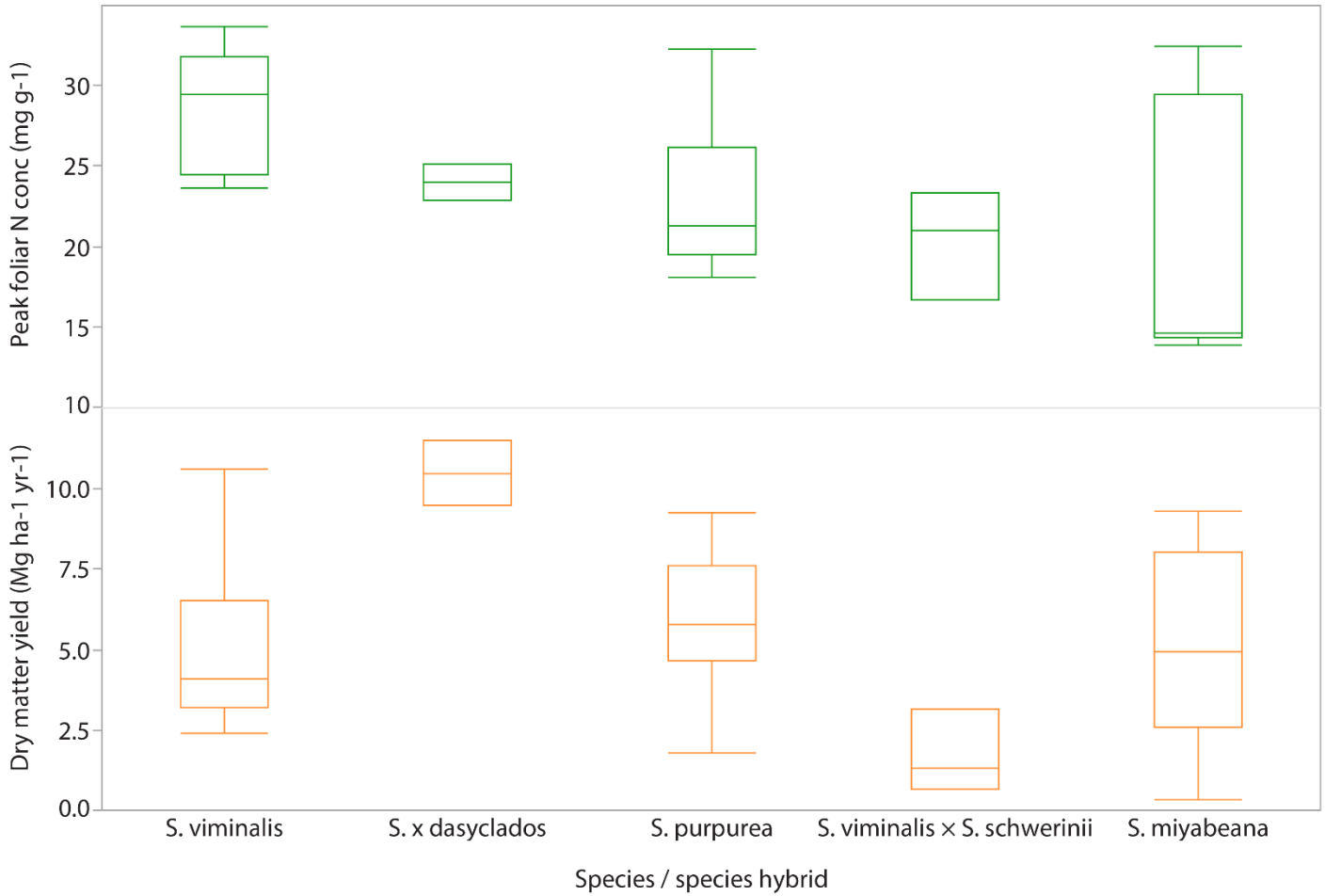


Figure 1.1 Box plot representation of foliar N concentration and dry stem biomass yield for six species or species hybrids reported in the literature.

1.3.3 Harvest removal of N

Values for stem biomass N concentration and biomass yield based on the literature review suggest that there are strong differences among species and species hybrids (Figure 1.2). This is further demonstrated with the calculation of stem biomass N use efficiencies (Figure 1.3). These data may suggest that newer *S. schwerinii* and *S. viminalis* hybrids have improved N use efficiency over the older pure *S. viminalis* cultivars. *Salix miyabeana* has a prominent role in the US breeding program, mainly through the creation of triploid hybrids with *S. viminalis* and *S. purpurea*, which have demonstrated improved yields over diploid cultivars (Fabio et al., 2017; Serapiglia et al., 2014) (See Chapters 2 and 3). Unfortunately, little data exist at this point on N use efficiency in these cultivars in field trials, although a recent pot study demonstrated that the top-yielding triploid hybrids do indeed have N use efficiencies that are significantly greater than current commercial cultivars (See Chapter 5).

Based on the N removal rates and yield ranges given as N replacement rates by various authorities (Table 1.1), the mean N content of biomass is 4.6 kg N Mg^{-1} removed at harvest on a dry matter basis, while the lowest value is 1.7 provided by Adegbidi et al. (2001), and the greatest value is 13.3 kg N Mg^{-1} provided by Caslin et al. (2010). The mean value based on the literature survey was 4.5 kg N Mg^{-1}, remarkably close to the average based on guidelines. The lowest value was 1.2 kg N Mg^{-1}, while the greatest was 7.4 kg N Mg^{-1}, suggesting that the upper value provided by Caslin et al. (2010) may be too high. Nonetheless, stem N content appears to be somewhat predictable for willow SRC in general, with perhaps somewhat predictable differences by species.

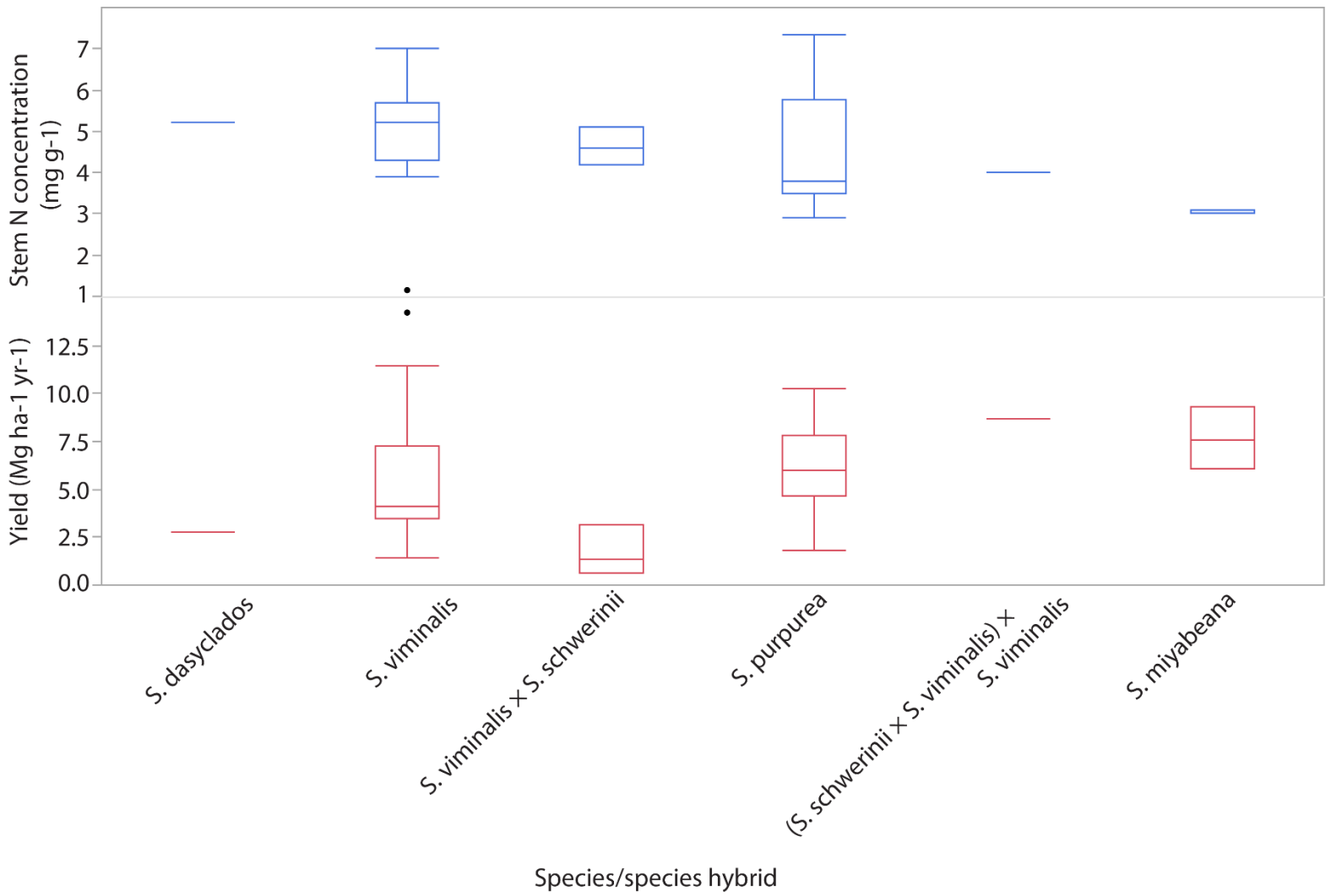


Figure 1.2 Box plot representation of stem N concentration and dry stem biomass yield for six species or species hybrids reported in the literature.

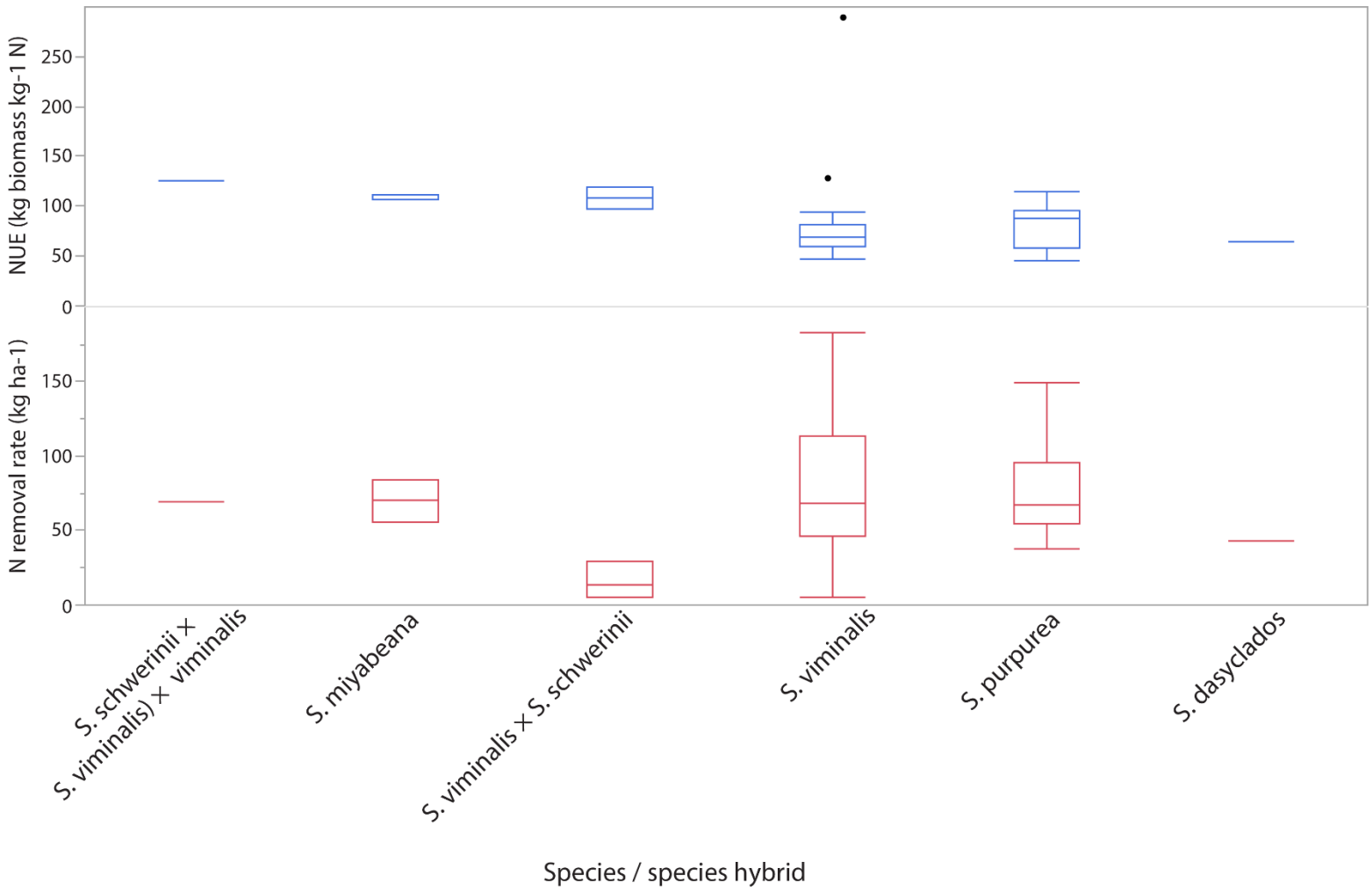


Figure 1.3 Box plot representation of stem N use efficiency and N removal rates for six species or species hybrids calculated from the literature.

1.3.4 Effects of N fertilization on biomass production

The literature search resulted in eight common SRC willow species or species hybrids with direct comparisons between fertilized and unfertilized control treatments (Figure 1.4). Fertilized treatment means had consistently greater absolute yields over control treatments for all species and hybrids. *Salix miyabeana* cultivars had the greatest overall yields, but the lowest percent difference between fertilized and control treatments. In contrast, *S. dasyclados* and *S.* × *dasyclados* cultivars had relatively low absolute yields, but the greatest response to fertilization over the control (201 and 142% respectively). *Salix schwerinii* × *S. viminalis* hybrids showed the next greatest percent increase in yield over the control, greater than the pure *S. viminalis* cultivars and the other the two *S. viminalis* hybrids. A plot of the percentage yield change data against the N fertilization treatment level (kg N ha^{-1}) shows no positive significant relationship (Figure 1.5). These results should be interpreted with caution, however, because although there were a relatively large number of observations, all cultivars did not necessarily experience the same environmental conditions or experimental treatments. Nord-Larsen et al. (2015) analyzed estimated yields from 25 commercial fields and together with site factors, found that well fertilized stands had significantly greater yields compared to stands that were never fertilized or fertilized just once.

There were 116 observations that involved synthetic fertilizer and 52 observations involving organic treatments across the 24 studies; however, there were only five studies that made direct comparisons among synthetic and organic fertilization and unfertilized control treatments (Table 1.2). Based on this relatively small set of studies there were no clear differences between fertilization types or among species, with both absolute yields and percent increase over the control very similar for organic and synthetic sources of N.

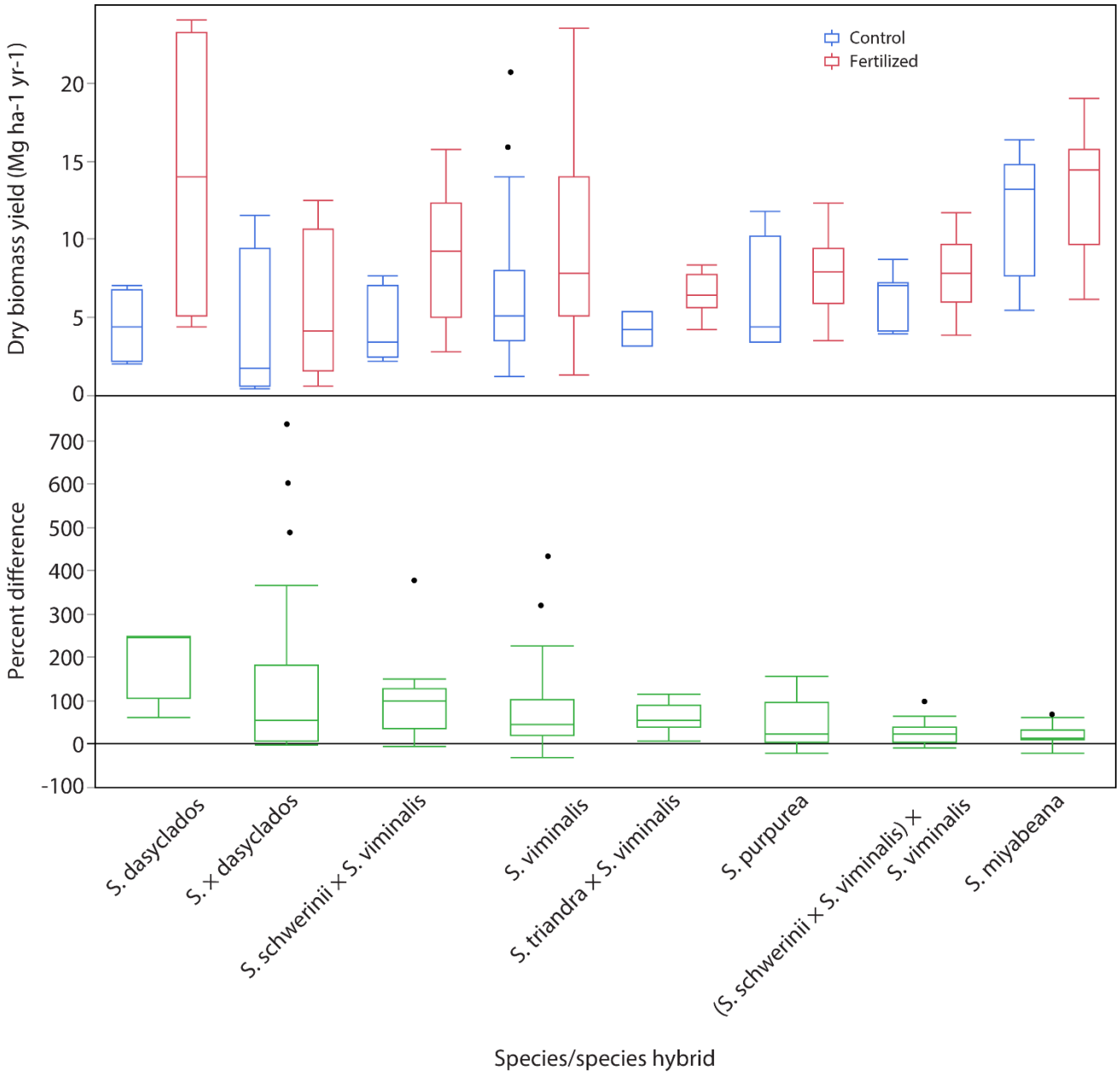


Figure 1.4 Box plots of control (blue), fertilizer treatment (red) and percent difference (green) in yields by shrub willow species or species hybrids.

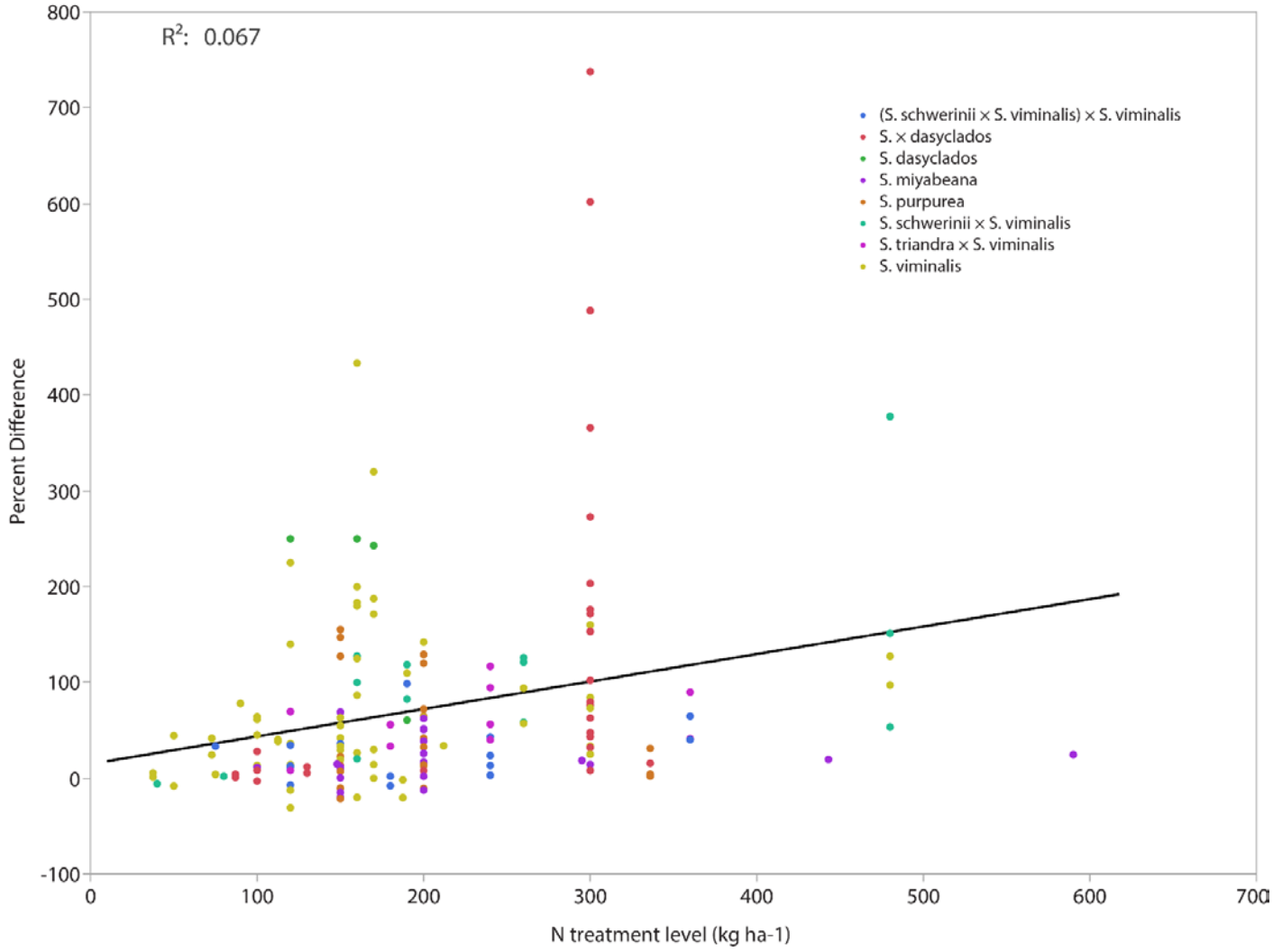


Figure 1.5 Percent difference between fertilization and control treatments across all observations collected from the literature search.

Marron (2015) reviewed different organic amendment treatments for three SRC species, including willow, and found overall a mostly positive effect of organic amendments on growth, with sewage sludge having the greatest positive effect, followed by manure. One advantage ascribed to organic amendments is that they potentially act as a slow-release source of N (Adegbidi and Briggs, 2003; Adegbidi et al., 2003), which would be more advantageous in a non-experimental setting where growth will most likely preclude the use of machinery for application. The application of waste water or manure to SRC plantations been advocated for some time as a way to deal with waste products with high nutrient contents (Aronsson and Perttu, 2001; Guidi Nissim et al., 2015; Kuzovkina and Quigley, 2005).

Table 1.3 Experimental comparisons between organic and synthetic sources of N fertilizer represented as absolute mean annual yields and percent yield gain compared to the control treatment.

		Yield (Mg ha^{-1} yr^{-1})		**Percent over control**	
Reference	**Species/ species hybrid**	**Organic**	**Synthetic**	**Organic**	**Synthetic**
Cavanagh et al. (2011)	*S. miyabeana*	15.8	15.1	19.4	14.3
Larsen et al. (2016)	(*S. schwerinii* × *S. viminalis*) × *S. viminalis*	6.5	6.6	55.1	64.3
	S. triandra × *S. viminalis*	7.0	6.4	24.8	14.8
Quaye & Volk (2013)	*S. miyabeana*	7.8	7.9	47.8	53.8
	S. purpurea	12.2	11.8	23.2	15.7
Quaye et al. (2011)	*S.* × *dasyclados*	11.1	10.7	5.6	2.7
Sevel et al. (2014)	(*S. schwerinii* × *S. viminalis*) × *S. viminalis*	9.7	11.7	11.5	34.5
Mean		10.0	9.1	31.3	32.5

1.3.5 Multi-rotational fertilization effects

Given the fact that multiple harvests will lead to cumulative nutrient removal based on the concentration in biomass and that yields after the first rotation are expected to remain constant or increase (Sleight and Volk, 2016; Sleight et al., 2016), it could be assumed that a stronger yield response to fertilization would occur in later rotations compared to an unfertilized

control. Long-term research on fertilization effects with sewage sludge over four, three- to four-year rotations in eastern Canada Guidi Nissim et al. (2013) found that the yield response remained relatively constant expressed as percent increase between 34 and 45%. There was no clear temporal trend across the four rotations and fluctuations were likely due to weather conditions. Recently published work from Denmark indicated that fertilization over three consecutive two-year rotations for both synthetic and organic fertilizers stimulated yields over the control at the lowest levels, however, in the second rotation yields decreased in the higher fertilization levels (Georgiadis et al., 2017). There do not appear to be any published results from fertilization trials in the US that have occurred continuously over multiple three-year rotations.

1.4 Environmental and economic considerations

1.4.1 N losses through nitrate leaching

A relatively small number of studies have reported nitrate leaching measurements, however, a quantitative analysis using data from the literature was difficult because of differences in the way that nitrate is reported. For instance, some studies reported nitrate as mg N L^{-1}, while others report leaching losses as kg N ha^{-1} yr^{-1}. Also, some studies report concentrations at each sampling time point, while others report mean concentrations across study periods. Despite these discrepancies, there is, perhaps not surprisingly, evidence that fertilization of newly planted willow can result in increased leaching losses. For instance, Balasus et al. (2012) found that fertilization of newly established *S. viminalis* in Germany at a level of 75 kg N ha^{-1} yr^{-1} as calcium nitrate resulted in leaching losses constituting between 23 and 49% of applied N in first and second years of growth. These levels were significantly greater than those measured under 50 kg N ha^{-1} yr^{-1} which were similar to the unfertilized control treatment. Mortensen et al. (1998)

found that fertilization at a level of 75 kg N ha^{-1} also significantly increased leaching losses at two sites in Denmark, although this effect was only measured in the first year of growth. Labrecque and Teodorescu (2001), found significantly elevated nitrate concentrations at two sites fertilized with sewage sludge. Dimitriou et al. (2012) sampled ground water nitrate concentrations across 16 willow fields, some of which had received sewage sludge and some of which had not. Instead of comparing to an unfertilized control field, the authors compared concentrations in willow to those measured in a reference, which was typically planted with cereals or in grass cover. They found that mean reference field nitrate concentrations were close to 5 mg NO_3-N L^{-1}, which was an order of magnitude greater than concentrations in willow fields. They could not find evidence that sludge application caused any increase in nitrate concentrations, which is in contrast to the findings of Labrecque and Teodorescu (2001).

A significant source of nitrate leaching is likely from soil disturbance at the time of plantation establishment, when the willow cuttings have not developed a root system to capture dissolved nutrients (Goodlass et al., 2007), especially when compared to well-established SRC plantations (Schmidt-Walter and Lamersdorf, 2012). Moukoumi et al. (2012) measured significantly lower soil available nitrate levels in the second year after establishment compared to the first year in locations with higher soil N stocks, however, they did not estimate leaching losses. There has also been speculation that harvesting may result in significant N losses, although this has not been confirmed through field experimental measurements (Aronsson et al., 2000; Dimitriou et al., 2012).

1.4.2 Estimation of net worth of fertilizer effects

Another obvious question is whether or not any increase in biomass production through the application of fertilizer has a net economic benefit. Aronsson et al. (2014) tested the

effectiveness of three fertilization management strategies ranging in quantity and frequency of nutrients applied. They found that higher fertilization levels in general led to greater net values of biomass given a net value of 41.3 € per Mg of dry biomass and a fertilizer cost of 0.9 € per kg N. Through a sensitivity analysis they demonstrated that at lower biomass net values or higher fertilizer costs, the lower intensity fertilization treatments can change the conclusion. The net value of biomass and cost of N fertilizer from Aronsson et al. (2014) were applied to the net biomass change between fertilized and control treatments for the studies in this literature review that used synthetic fertilizer. There was a large amount of variation in net worth across the range of cumulative fertilizer levels applied in the studies from this literature review (Figure 1.6). When viewed individually, there were only a few studies that had entirely positive net worth as a result of fertilization treatments, while most of the studies spanned a range of positive and negative net worth (Figure 1.7). Applying fixed prices uniformly across this wide array of studies does not represent a realistic scenario, since they span a range of over 20 years and include studies from numerous countries where prices and materials differ widely. This was done merely for illustrative purposes. It does also invite the question of whether the use of organic sources of fertilization could be more beneficial from an economic stand point, since the performance of willow under organic and synthetic treatments were similar (Table 1.2). Some of the organic fertilizer sources found in this literature review include materials that are often considered waste products and may otherwise incur a tipping fee for disposal, however the cost of transport would be a significant factor due to the low nutrient densities of these materials.

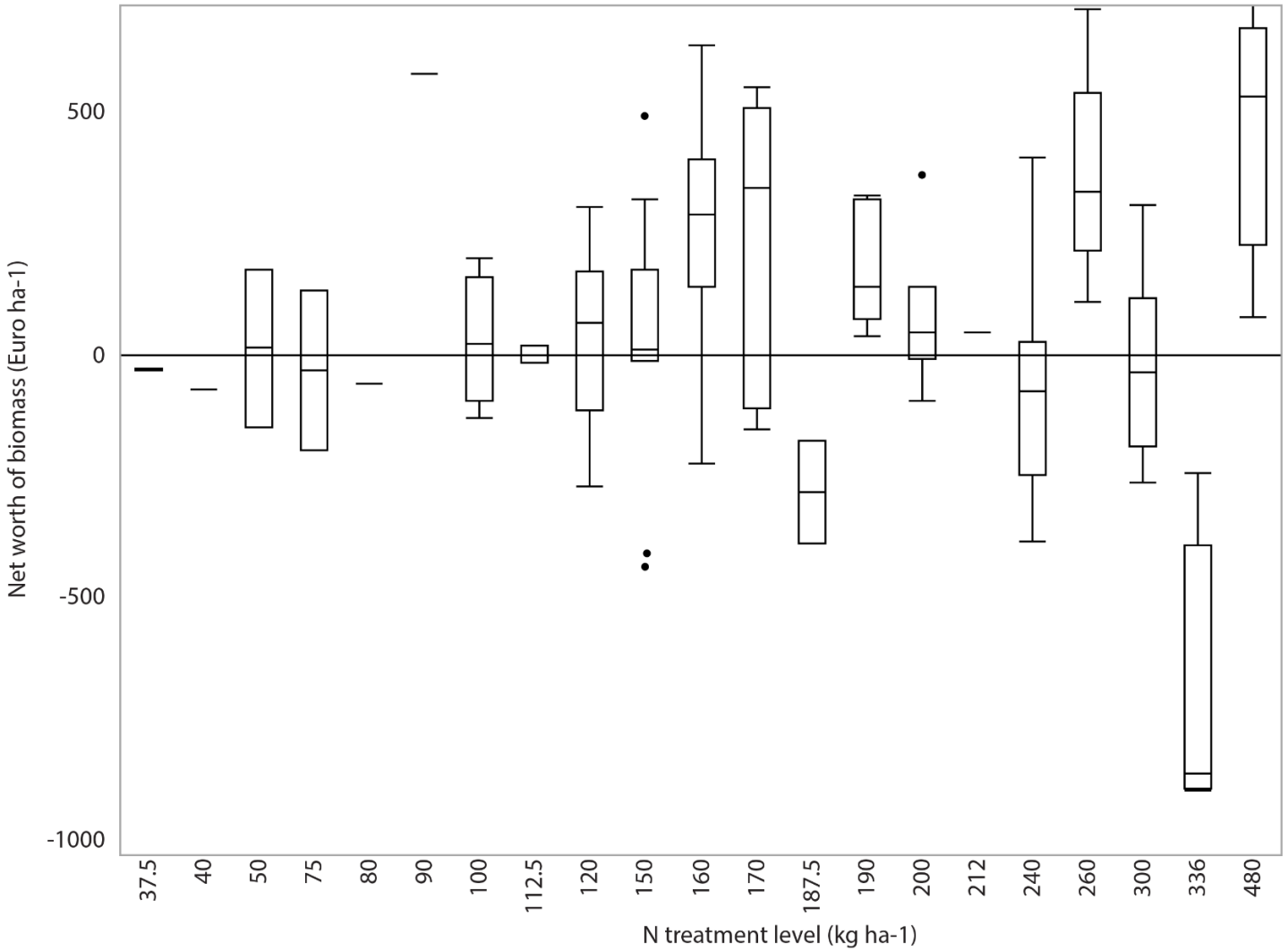


Figure 1.6 Net worth of biomass produced through fertilization by synthetic fertilizer rate using economic parameters from Aronsson et al. (2014).

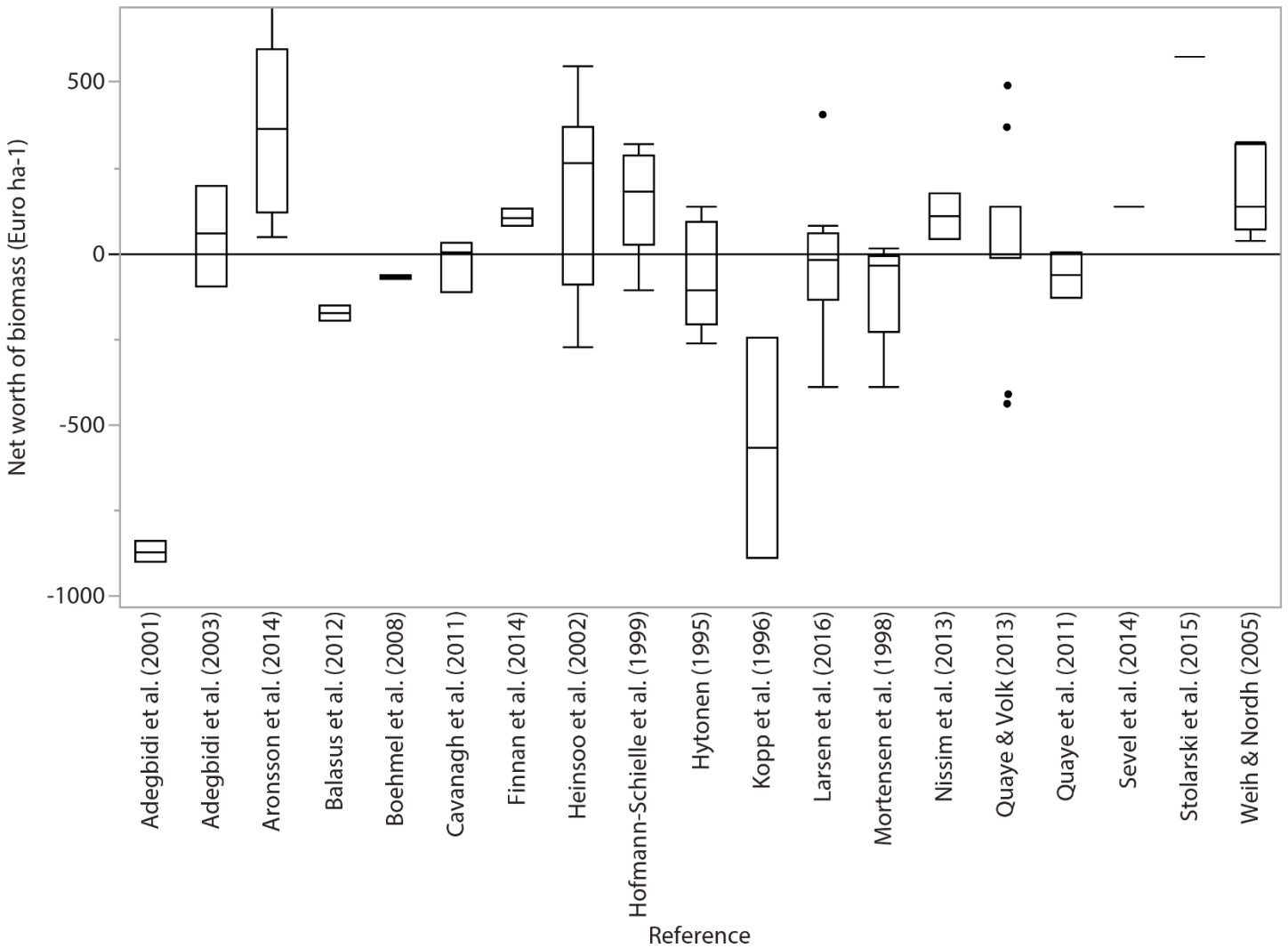


Figure 1.7 Net worth of biomass produced through fertilization by study using only synthetic fertilizer and economic parameters used by Aronsson et al. (2014).

Regardless, it is expected that their application to willow in place of synthetic fertilizers will have net economic and environmental benefits (Heller et al., 2003).

1.5 Conclusions and recommendations

Overall these studies have shown that fertilization of willow can improve yields, but there were a few observations with neutral or somewhat negative effects. While the response is for the most part positive, it also seems to be highly influenced by site factors, and therefore it is difficult at this point to say definitively whether or not it is warranted as a means to improve yields in an economically and environmentally sound manner. While site-specific yield response to fertilization is difficult to predict with the set of studies reviewed here, it is definitive that N removal rate due to harvest is quantifiable and scales with yield since N concentrations in willow stem biomass are fairly predictable. Biomass sampling for nutrient content at harvest should become a routine practice, while more studies should focus on capturing the opportunity to test whether or not long-term stand N depletion occurs and if this negatively impacts long-term yields. The suggestion by Aronsson et al. (2014) that producers should fertilize one portion of their field in order to compare yields to non-fertilized yields to determine a local need for added nutrients seems practical, especially when coupled with precision measurements of yield across fields. These types of “on-farm” strip trials, when performed with the coordination of agricultural extension professionals across many fields have the potential to provide valuable agronomic data. Examples of national or international coordinated efforts for plant material testing exist for important food crops (Smith et al., 2001). Collaborations between willow breeding programs have occurred in both Europe and North America, but mainly in the form of exchange of plant material (Zsuffa, 1990), but some transatlantic coordinated research efforts have produced some interesting genotype-by-environment interactions. For instance, a cultivar

from the UK breeding program was identified as a top-producer in a recent multi-environment trial analysis in the US (Fabio et al., 2017) (See Chapter 3), while some US cultivars performed well in the UK (A. McCracken et al., unpublished data). Long-term, coordinated trials with the same treatment structure, sampling design and site characterization requirements could help to better define nutrient recommendations. Leveraging existing field trials for the initiation of longer-term, multi-rotation fertilizer response trials may also benefit this area of research. The use of unmanned aerial systems equipped with multi-spectral sensors and Light Detection and Ranging technology could be used to estimate canopy N and biomass. This technology coupled with precision agriculture equipment, such as yield monitors and variable rate fertilizer applicators has the potential to greatly improve crop management capabilities.

1.6 References

Abrahamson LP, Volk TA, Smart LB, Cameron KD. Shrub willow biomass producer's handbook. http://willow.cals.cornell.edu/extension/producers-handbook/ (Last accessed June 29, 2017

Adegbidi HG, Briggs RD (2003) Nitrogen mineralization of sewage sludge and composted poultry manure applied to willow in a greenhouse experiment. Biomass Bioenerg. 25:665-673.

Adegbidi HG, Briggs RD, Volk TA, White EH, Abrahamson LP (2003) Effect of organic amendments and slow-release nitrogen fertilizer on willow biomass production and soil chemical characteristics. Biomass Bioenerg. 25:389-398.

Adegbidi HG, Volk TA, White EH, Abrahamson LP, Briggs RD, Bickelhaupt DH (2001) Biomass and nutrient removal by willow clones in experimental bioenergy plantations in

New York State. Biomass Bioenerg. 20:399-411.

Agriculture and Horticulture Development Board. Nutrient Management Guide (RB209). http://www.ahdb.org.uk/projects/RB209.aspx (Last accessed June 29, 2017

Albertsson J, Verwijst T, Hansson D, Bertholdsson NO, Åhman I (2014) Effects of competition between short-rotation willow and weeds on performance of different clones and associated weed flora during the first harvest cycle. Biomass Bioenerg. 70:364-372.

Amichev BY, Hangs RD, Konecsni SM, Stadnyk CN, Volk TA, Bélanger N, Vujanovic V, Schoenau JJ, Moukoumi J, Van Rees KCJ (2014) Willow Short-Rotation Production Systems in Canada and Northern United States: A Review. Soil Sci Soc Am J. 78:S168-S182.

Aronsson P, Dahlin T, Dimitriou I (2010) Treatment of landfill leachate by irrigation of willow coppice – Plant response and treatment efficiency. Environ Pollut. 158:795-804.

Aronsson P, Perttu K (2001) Willow vegetation filters for wastewater treatment and soil remediation combined with biomass production. Forest Chron. 77:293-299.

Aronsson P, Rosenqvist H, Dimitriou I (2014) Impact of nitrogen fertilization to short-rotation willow coppice plantations grown in Sweden on yield and economy. Bioenerg Res. 7:993-1001.

Aronsson PG, Bergstrom LF, Elowson SNE (2000) Long-term influence of intensively cultured short-rotation Willow Coppice on nitrogen concentrations in groundwater. J. Environ. Manage. 58:135-145.

Balasus A, Bischoff WA, Schwarz A, Scholz V, Kern J (2012) Nitrogen fluxes during the initial stage of willows and poplars in short-rotation coppices. J. Plant Nutr. Soil Sci. 175:729-738.

Berhongaray G, Verlinden MS, Broeckx LS, Ceulemans R (2015) Changes in belowground biomass after coppice in two Populus genotypes. For. Ecol. Manage. 337:1-10.

Bollmark L, Sennerby-Forsse L, Ericsson T (1999) Seasonal dynamics and effects of nitrogen supply rate on nitrogen and carbohydrate reserves in cutting-derived *Salix viminalis* plants. Can J For Res. 29:85-94.

Brereton NJB, Pitre FE, Shield I, Hanley SJ, Ray MJ, Murphy RJ, Karp A (2014) Insights into nitrogen allocation and recycling from nitrogen elemental analysis and 15N isotope labelling in 14 genotypes of willow. Tree Physiol. 34:1252-1262.

Caslin B, Finnan J, McCracken A. Short rotation willow coppice guidelines. Teagasc ISBN 1-84170-568-3. http://www.agriculture.gov.ie/media/migration/ruralenvironment/environment/bioenergyscheme/WillowBestPracticeManual190913.pdf. (Accessed June 30, 2017)

Cerasuolo M, Richter GM, Richard B, Cunniff J, Girbau S, Shield I, Purdy S, Karp A (2016) Development of a sink–source interaction model for the growth of short-rotation coppice willow and in silico exploration of genotype×environment effects. J Exp Bot. 67:961-977.

Dimitriou I, Mola-Yudego B, Aronsson P (2012) Impact of Willow Short Rotation Coppice on Water Quality. Bioenerg Res. 5:537-545.

Djomo SN, Kasmioui OE, Ceulemans R (2011) Energy and greenhouse gas balance of bioenergy production from poplar and willow: a review. GCB Bioenergy. 3:181-197.

Ens J, Farrell RE, Belanger N (2013) Effects of edaphic conditions on site quality for *Salix purpurea* 'Hotel' plantations across a large climatic gradient in Canada. New For. 44:899-918.

Ericsson T (1994) Nutrient cycling in energy forest plantations. Biomass Bioenerg. 6:115-121.

Ericsson T, Rytter L, Linder S (1992) Nutritional dynamics and requirements of short rotation forests. In: Mitchell CP, Ford-Robertson JB, Hinckley TM, Sennerby-Forsse L (eds) Ecophysiology of short rotation forest crops. Elsevier Applied Science, London, pp 32-65.

Fabio ES, Kemanian AR, Montes F, Miller RO, Smart LB (2017) A mixed model approach for evaluating yield improvements in interspecific hybrids of shrub willow, a dedicated bioenergy crop. Ind Crops Prod. 96:57-70.

Georgiadis P, Sevel L, Raulund-Rasmussen K, Stupak I (2017) Fertilization of Willow Coppice Over Three Consecutive 2-Year Rotations—Effects on Biomass Production, Soil Nutrients and Water. Bioenerg Res

Goodlass G, Green M, Hilton B, McDonough S (2007) Nitrate leaching from short-rotation coppice. Soil Use Manag. 23:178-184.

Guidi Nissim W, Jerbi A, Lafleur B, Fluet R, Labrecque M (2015) Willows for the treatment of municipal wastewater: Performance under different irrigation rates. Ecol. Eng. 81:395-404.

Guidi Nissim W, Pitre FE, Teodorescu TI, Labrecque M (2013) Long-term biomass productivity of willow bioenergy plantations maintained in southern Quebec, Canada. Biomass Bioenerg. 56:361-369.

Guidi W, Pitre FE, Labrecque M (2013) Short-rotation coppice of willows for the production of biomass in eastern Canada. In: Matovic MD (ed) Biomass Now—Sustainable Growth and Use. In Tech Open Science, pp 421-448.

Hangs RD, Schoenau JJ, Van Rees KCJ, Bélanger N, Volk T (2014a) Leaf Litter Decomposition

and Nutrient-Release Characteristics of Several Willow Varieties Within Short-Rotation Coppice Plantations in Saskatchewan, Canada. Bioenerg Res. 7:1074-1090.

Hangs RD, Schoenau JJ, Van Rees KCJ, Bélanger N, Volk T, Jensen T (2014b) First Rotation Biomass Production and Nutrient Cycling within Short-Rotation Coppice Willow Plantations in Saskatchewan, Canada. Bioenerg Res. 7:1091-1111.

Hangs RD, Schoenau JJ, Van Rees KCJ, Knight JD (2012) The effect of irrigation on nitrogen uptake and use efficiency of two willow (Salix spp.) biomass energy varieties. Can J Plant Sci. 92:563-575.

Hauk S, Knoke T, Wittkopf S (2014) Economic evaluation of short rotation coppice systems for energy from biomass—A review. Renewable and Sustainable Energy Reviews. 29:435-448.

Heller MC, Keoleian GA, Volk TA (2003) Life cycle assessment of a willow bioenergy cropping system. Biomass Bioenerg. 25:147-165.

Hytönen J (1995) Effect of fertilizer treatment on the biomass production and nutrient uptake of short-rotation willow on cut-away peatlands. Silva Fenn. 29:21-40.

Jug A, Hofmann-Schielle C, Makeschin F, Rehfuess KE (1999) Short-rotation plantations of balsam poplars, aspen and willows on former arable land in the Federal Republic of Germany. II. Nutritional status and bioelement export by harvested shoot axes. For. Ecol. Manage. 121:67-83.

Kopinga J, Van den Burg J (1995) Using soil and foliar analysis to diagnose the nutritional status of urban trees. J. Arboric. 21:17-17.

Kuzovkina YA, Quigley MF (2005) Willows beyond wetlands: Uses of Salix L. species for environmental projects. Water Air Soil Pollut. 162:183-204.

Labrecque M, Teodorescu TI (2001) Influence of plantation site and wastewater sludge fertilization on the performance and foliar nutrient status of two willow species grown under SRIC in southern Quebec (Canada). Forest Ecology and Management. 150:223-239.

Larsen SU, Jørgensen U, Kjeldsen JB, Lærke PE (2016) Effect of fertilisation on biomass yield, ash and element uptake in SRC willow. Biomass Bioenerg. 86:120-128.

Marron N (2015) Agronomic and environmental effects of land application of residues in short-rotation tree plantations: A literature review. Biomass Bioenerg. 81:378-400.

Mitchell CP, Stevens EA, Watters MP (1999) Short-rotation forestry – operations, productivity and costs based on experience gained in the UK. For. Ecol. Manage. 121:123-136.

Mortensen J, Nielsen KH, Jorgensen U (1998) Nitrate leaching during establishment of willow (Salix viminalis) on two soil types and at two fertilization levels. Biomass Bioenerg. 15:457-466.

Moukoumi J, Farrell RE, Van Rees KJC, Hynes RK, Bélanger N (2012) Intercropping *Caragana arborescens* with *Salix miyabeana* to Satisfy Nitrogen Demand and Maximize Growth. Bioenerg Res. 5:719-732.

Nord-Larsen T, Sevel L, Raulund-Rasmussen K (2015) Commercially grown short rotation coppice willow in Denmark: Biomass production and factors affecting production. Bioenerg Res. 8:325-339.

Richards BK, Stoof CR, Cary IJ, Woodbury PB (2014) Reporting on marginal lands for bioenergy feedstock production: a modest proposal. Bioenerg Res. 7:1060-1062.

Rohatgi A. WebPlotDigitizer V 3.12. http://arohatgi.info/WebPlotDigitizer (Accessed July 1, 2017).

Schmidt-Walter P, Lamersdorf NP (2012) Biomass production with willow and poplar short rotation coppices on sensitive areas—the impact on nitrate leaching and groundwater recharge in a drinking water catchment near Hanover, Germany. Bioenerg Res. 5:546-562.

Serapiglia MJ, Gouker FE, Smart LB (2014) Early selection of novel triploid hybrids of shrub willow with improved biomass yield relative to diploids. BMC Plant Biol. 14:74.

Sevel L, Nord-Larsen T, Ingerslev M, Jørgensen U, Raulund-Rasmussen K (2014) Fertilization of SRC Willow, I: Biomass Production Response. Bioenerg Res. 7:319-328.

Simon M, Zsuffa L, Burgess D (1990) Variation in N, P, and K status and N efficiency in some North American willows. Can J For Res. 20:1888-1893.

Sleight NJ, Volk TA (2016) Recently Bred Willow (Salix spp.) Biomass Crops Show Stable Yield Trends Over Three Rotations at Two Sites. Bioenerg Res:1-16.

Sleight NJ, Volk TA, Johnson GA, Eisenbies MH, Shi S, Fabio ES, Pooler PS (2016) Change in Yield Between First and Second Rotations in Willow (Salix spp.) Biomass Crops is Strongly Related to the Level of First Rotation Yield. Bioenerg Res. 9:270-287.

Smith A, Cullis B, Gilmour A (2001) Applications: The analysis of crop variety evaluation data in Australia. Aust N Z J Stat. 43:129-145.

Stoof CR, Richards BK, Woodbury PB, Fabio ES, Brumbach AR, Cherney J, Das S, Geohring L, Hansen J, Hornesky J, Mayton H, Mason C, Ruestow G, Smart LB, Volk TA, Steenhuis TS (2015) Untapped potential: Opportunities and challenges for sustainable bioenergy production from marginal lands in the Northeast USA. Bioenerg Res. 8:482-501.

Weih M, Ronnberg-Wastljung AC (2007) Shoot biomass growth is related to the vertical leaf nitrogen gradient in Salix canopies. Tree Physiol. 27:1551-1559.

Zsuffa L (1990) Genetic improvement of willows for energy plantations. Biomass. 22:35-47.

CHAPTER 2

Genotype by environment interactions analysis of North American shrub willow yield trials confirms superior performance of triploid hybrids

GCB Bioenergy (2017) 9:445-459

2.1 Abstract

Development of dedicated bioenergy crop production systems will require accurate yield estimates, which will be important for determining many of the associated environmental and economic impacts of their production. Shrub willow (*Salix* spp.) is being promoted in areas of the US and Canada due to its adaption to cool climates and wide genetic diversity available for breeding improvement. Willow breeding in North America is in an early stage and selection of elite genotypes for commercialization will require testing across broad geographic regions to gain an understanding of how shrub willow interacts with the environment. A dataset of first rotation shrub willow yields of 16 genotypes across 10 trial environments in the US and Canada was analyzed for genotype by environment interactions using the Additive Main effects and Multiplicative Interactions (AMMI) model. Mean genotype yields ranged from 5.22 to 8.58 oven dry Mg ha^{-1} yr^{-1}. Analysis of the main effect of genotype showed that one round of breeding improved yields by as much as 20% over check cultivars, and that triploid hybrids, most notably *Salix viminalis* × *S. miyabeana*, exhibited superior yields. Important variability was found in genotypic response to environments, which suggests specific adaptability could be exploited among 16 genotypes for yield gains. Strong positive correlations were found between environment main effects and AMMI interaction parameters and growing environment temperatures. These findings demonstrate yield improvements are possible in one generation, and will be important for developing cultivar recommendations and for future breeding efforts.

2.2 Introduction

If dedicated bioenergy crops are to play a significant role in climate change mitigation strategies, a clear understanding of yield potential on a regional basis must be established. Development and delivery of high-yielding, well-adapted crops is a key underlying assumption in the estimation of bioenergy production capacity in the US (U. S. Department of Energy, 2011). Crop management and breeding will play crucial roles in meeting the challenges of producing more biomass on limited land in a sustainable manner (Karp & Shield, 2008). Shrub willow (*Salix* spp.) grown in short rotation has shown promise as a viable, regionally based feedstock for marginal land due to its adaptability to cool, moist climates with short growing seasons (Kuzovkina & Quigley, 2005) and large potential for genetic improvement through breeding (Smart et al., 2005; Smart & Cameron, 2008). This substantial, yet mostly underexploited genetic variability among taxa, is expected to provide a basis for developing key traits desirable for sustainable bioenergy production, both through traditional breeding and with advanced molecular techniques (Karp et al., 2011).

Willow breeding for biomass production has been most extensively researched in the UK (Lindegaard & Barker, 1997) and Sweden (Larsson, 1998). A thorough account of the global breeding history of shrub willow is provided by Kuzovkina et al. (2008). In North America, shrub willow breeding efforts began at the University of Toronto in the early 1980's with collection of native species and exchange of plant material with the UK and European countries (Zsuffa, 1990), but efforts were focused largely on hybridizations between North American native species (Mosseler, 1990). In the US, acquisition of plant material from collaborators in Canada, as well as China, Japan, New Zealand, Ukraine and Sweden provided the basis of a breeding program focused largely on novel interspecific hybridizations displaying heterosis for

biomass yield traits (Kopp et al., 2001; Smart et al., 2005; Smart & Cameron, 2012). Polyploidy is common in *Salix* and novel triploid species hybrids have been linked to improved yields and biomass quality in early selection trials (Smart et al., 2008; Serapiglia et al., 2014; Serapiglia et al., 2015). Cultivar development, however, is a multistage screening process requiring substantial investments in time and resources prior to commercialization (Hanley & Karp, 2013). An accurate evaluation of cultivar yield potentials is ultimately assessed through testing on multiple sites with a diverse range of environmental characteristics.

Biomass yield is an important factor determining the environmental and economic impacts associated with growing shrub willow. Life cycle analyses have shown that yield assumptions can significantly impact net energy ratios and greenhouse gas balances (Heller et al., 2003; Keoleian & Volk, 2005; Caputo et al., 2014), as well as economic returns on investment and production costs (Buchholz & Volk, 2011; Hauk et al., 2014). There have been numerous research trials conducted over the past two decades in regions of the Northeastern US and Central and Eastern Canada that have quantified yields. Some studies have analyzed the differential response of genotype to contrasting environmental conditions (Labrecque & Teodorescu, 2003; Wang & MacFarlane, 2012; Serapiglia et al., 2013; Mosseler et al., 2014a); however, the limited number of test sites and use of diverse cultivars of various levels of genetic improvement makes it difficult to generalize specific genotypic responses to larger growing regions. Others have summarized mean yields from multiple test sites across geographical regions (Kiernan et al., 2003; Volk et al., 2011), but due to unequal representation of genotypes among trials, little insight into the genotypic contribution to yield variability can be gained. Furthermore, efforts to model yields across broad geographic ranges may use general yield estimates from obsolete cultivars or ones that may not be well adapted for a particular region

(Walsh et al., 2003; Wang et al., 2015). Plant breeders and agronomists are therefore challenged with assessing genotypic sensitivity to certain edaphic and climatic conditions. This process is also important for assuring continued improvement within a breeding program, and for providing a basis for recommending cultivars for broad scale production.

These recommendations become complicated when significant cross-overs occur in genotype (GEN) yield rankings, as a response to contrasting environments (ENV). These genotype by environment (G×E) interactions are prominent and important phenomena in agriculture, which present both challenges and opportunities for plant breeders and agronomists. Selection and deployment of elite cultivars must be based on results of rigorous testing through coordinated multi-location trials, combined with appropriate statistical analyses for assessing the adaptability of genotypes and predicting performance in untested ENV (Annicchiarico, 2002). A longstanding theme in plant breeding is to focus the search for GEN that exhibit stable yields or broad adaptability across ENV in the targeted growing region. This concept was popularized by Finlay & Wilkinson (1963) and Eberhart & Russell (1966), who developed regression parameters that seek to identify superior yielding GEN that maintain stable performance across ENV. Selection on the basis of stability can help to minimize the complicating effects of G×E interactions, adding efficiency to the selection process by focusing resources on material that has the best promise for widespread optimal performance (Eberhart & Russell, 1966). Selection based on stability should also guard against a potentially detrimental tendency to select GEN based on greater yields in only favorable ENV (Simmonds, 1991; Annicchiarico, 2002). However, when the G×E component is strong and meaningful, there is also a counter argument that contrasting performance among GEN can be capitalized upon, and breeding efforts should focus on specific adaptation (Cooper & Hammer, 1996; Piepho & Möhring, 2005). Thus G×E

interactions are viewed as a useful and informative aspect of cultivar testing, and sub-dividing a growing region into smaller areas and targeting GEN at those areas can improve overall yields (Gauch & Zobel, 1997). This argument is reinforced by the fact that much of the world's crop production occurs on land that is less favorable than that where the crops were developed (Simmonds, 1991; Gauch & Zobel, 1997). Since dedicated bioenergy crops are presumably targeted for marginal lands (Richards et al., 2014; Stoof et al., 2015), perhaps this concept is particularly relevant. The relative merits of these two perspectives, exploiting only broad adaptations or else both broad and specific adaptations, vary from case to case and depend substantially on the relative magnitudes of genotype main effects and G×E interaction effects. We present an analysis of G×E interactions in first rotation yields across a network of shrub willow yield trials in North America covering 16 GEN and 10 ENV. The objectives of this study were 1) to identify shrub willow genotypes with broad adaptability in biomass yield across target growing regions in North America, 2) analyze G×E interactions for identifying and characterizing specific adaptation of certain genotypes and 3) identify edaphic and climatic variables that are most closely associated with G×E interaction patterns. This represents the most comprehensive analysis of North American shrub willow yields to date and will serve as a basis for making cultivar-site matching recommendations and will inform future breeding efforts.

2.3 Materials and methods

2.3.1 Breeding material and yield trial network

Foundational breeding material used in developing the GEN tested in these yield trials was obtained from the University of Toronto and from accessions of native and naturalized species collected in the northeastern US and eastern Canada in the 1980's to mid-1990's (Kopp et al., 2001; Smart & Cameron, 2008). Crosses were performed between 1998 and 1999 at SUNY-ESF, and after initial family screening in field trials for biomass yield traits, a group of genetically diverse individuals were deployed in regional yield trials. Between 2005 and 2011, 23 trials were established mainly in the northeastern and Midwestern US and contained between six and 30 genotypes. In order to provide an unbiased comparison of genotype yields, we restricted our analysis to 16 cultivars (Table 2.1) that were all present in each of 10 environments (Table 2.2). The yield trials were planted between 2006 and 2009 and hosted by institutions located in six US states and two Canadian provinces and the cultivars have been placed into diversity groups based on pedigree. Trials were established and maintained generally in a consistent manner across sites according to a standardized protocol and methods followed those described in Serapiglia et al. (2013) and Volk et al. (2011). Conventional tillage was used to prepare the sites either in the fall or spring prior to planting, which generally occurred between May and June. All trials were planted by hand using dormant 25-cm cuttings sourced from nursery beds at the Tully Genetics Field Station of SUNY-ESF in Tully, NY. Trials were laid out in a double-row configuration with 1.52 m between double-rows, 0.76 m within the double-rows and 0.61 m between plants along the row, for a planting density of 14,400 plants ha^{-1}. Within each yield trial genotype was the experimental treatment and the experimental units were plots consisting of three double rows, each 13 plants long, with the outer double-rows serving as

border rows. Genotypes were replicated four times in a randomized complete block design. Pre-emergence herbicides, generally oxyfluorfen (1.1 kg ai ha^{-1}) and simazine (2.2 kg ai ha^{-1}), were applied prior to budbreak, except at Boisbriand, QC, where no herbicide was used. Periodic mechanical or spot chemical weed control was performed as needed. After the first year of growth all stems were cut back during dormancy to promote coppice regrowth the following spring, at which time 112 kg N ha^{-1} was applied, normally as ammonium sulfate, except for the trial located in Saskatoon, SK where no fertilizer was applied. Harvests were conducted three years post-coppice (four-year-old root systems) during dormancy, except at Brimley, MI, which was harvested in July at 3.5 years post-coppice due to extreme moisture conditions in the field. Two to four plants on each end of the middle double row of each plot were excluded from harvest measurements to avoid plot-to-plot edge effects, resulting in 18-22 plants available for measurements. All stems from the 18-22 plants from the middle double row of each plot were cut with brush saws or cut and chipped with a mechanical harvester and weighed in a bin with weigh cells in the field. A subsample from each plot consisting of either whole stems or chips was collected, weighed fresh, dried at 65^{o}C to a constant weight and re-weighed to determine moisture content. Moisture content was used to calculate dry matter yield for each plot based on the area occupied by the harvested plants across a three-year rotation. All yields reported here are expressed as oven dried Mg ha^{-1} yr^{-1}. Survival data was also recorded on the harvested plants at the time of harvest, except Boisbriand, QC, where no survival data was collected, but survival was greater than 90% for each plot (M. Labrecque, personal observation) and Savoy, IL where survival was assessed in the middle of the second rotation, but most of the mortality occurred early in the first rotation (G. Kling, personal observation).

Table 2.1 Description of the 16 cultivars included in the genotype × environment interactions analysis of first rotation shrub willow yields.

Clone ID	Epithet	Species/pedigree	Mother	Father	Diversity group*	Sex	Ploidy†	Source
99239-015	'Allegany'	*S. koriyanagi* × *S. purpurea*	SH3	95058	6b	F	2X	Bred
9970-036	'Canastota'	*S. miyabeana*	SX61	SX64	5	M	4X	Bred
99202-004	'Fabius'	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X	Bred
9882-34	'Fish Creek'	*S. purpurea*	94006	94001	6a	M	2X	Bred
99217-015	'Millbrook'	*S. purpurea* × *S. miyabeana*	95026	SX64	9	F	3X	Bred
9980-005	'Oneida'	*S. purpurea* × *S. miyabeana*	94006	SX67	9	M	3X	Bred
99113-012	'Onondaga'	*S. koriyanagi* × *S. purpurea*	SH3	94002	6b	M	2X	Bred
99201-007	'Otisco'	*S. viminalis* × *S. miyabeana*	SV2	SX64	8	F	3X	Bred
99207-018	'Owasco'	*S. viminalis* × *S. miyabeana*	SV7	SX64	8	F	3X	Bred
S25	'S25'	*S. eriocephala*			4	F	2X	Bred
9871-31	'Sherburne'	*S. miyabeana*	SX61	SX67	5	F	4X	Bred
SV1	'SV1'	*S.* × *dasyclados*			1	F	2X	Unknown‡
SX61	'SX61'	*S. miyabeana*			5	F	4X	Natural accession
SX64	'SX64'	*S. miyabeana*			5	M	4X	Natural accession
99207-020	'Truxton'	*S. viminalis* × *S. miyabeana*	SV7	SX64	8	M	3X	Bred
99202-011	'Tully Champion'	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X	Bred

*Diversity group refers to Species/Pedigree

†Ploidy level estimated by flow cytometry (Serapiglia et al., 2015)

‡Collected in Ontario Canada, but possibly the cultivated hybrid *S. viminalis* × (*S. caprea* × *S. cinerea*) (Stott, 1991)

Table 2.2 Yield trial location characteristics. Precipitation, temperature and solar radiation data are means across four years of first rotation.

Location	Code	Year planted	Latitude	Longitude	Elevation (m)	Annual precip. (mm)	Annual GDD* (base 10°C)	Mean Annual $Temp_{min}$ (°C)	Solar radiation (MJ m^{-1} day^{-1})	SOM† (%)	Soil pH	Water table depth (cm)
Saskatoon, SK	Sask	2007	52.13	-106.61	510	408	767	-4.1	4329	4.5	7.07	200
Constableville, NY	Cons	2006	43.56	-75.53	513	1457	812	-1.7	4606	8.2	5.66	45
Skandia, MI	Skan	2009	46.36	-87.25	287	822	870	0.1	4909	3.6	6.47	30
Escanaba, MI	Esca	2008	45.77	-87.20	222	714	1016	-0.1	5147	2.8	6.10	200
Brimley, MI	Brim	2009	46.40	-84.47	200	791	1021	1.7	4980	4.0	5.25	15
Boisbriand, QC	Bois	2007	45.63	-73.89	30	1038	1162	1.2	4612	4.0	6.09	--
Middlebury, VT	Midd	2007	44.01	-73.20	114	1138	1419	2.0	4612	6.8	6.70	30
Waseca, MN	Wase	2006	44.06	-93.54	349	843	1459	1.7	4944	5.7	5.40	30
Fredonia, NY	Fred	2008	42.44	-79.29	255	909	1477	4.9	5082	3.6	4.80	30
Storrs, CT	Stor	2009	41.80	-72.23	198	1274	1487	5.4	5171	3.4	6.12	45

*GDD, growing degree days; †SOM, soil organic matter

2.3.2 Site environmental characteristics

Daily temperature and precipitation data for all four years of the harvest cycle were obtained from weather stations nearest to each trial location with the most complete records using publically accessible databases, National Oceanic and Atmospheric Agency, National Centers for Environmental Information (NOAA NCEI, 2015) for US trials, and Canadian National Climate Data (Environment Canada, 2015) for CA trials. Daily solar radiation estimates at a 1^{o} by 1^{o} grid scale were obtained from National Aeronautics and Space Adminstration, Prediction of Worldwide Energy Resource (NASA POWER, 2015). Soil samples were generally collected at the time of planting, or occasionally soon after harvest. Due to some differences between soil extraction methods, only pH (1:1 soil:water by weight) and % organic matter (typically by loss on ignition) were considered for data analysis.

2.3.3 Dataset refinement

Initially our dataset was comprised of 640 independent observations where the harvested plot was the experimental unit. While overall mean survival was greater than 90% across the yield trial network, some trials and individual plots experienced greater mortality. Two trials, Escanaba, MI and Saskatoon, SK, had mean survival values below 80%. Damage from herbicide drift appeared to be the main cause of mortality in Saskatchewan (Amichev et al., 2015), while at Escanaba mortality could not be associated with any particular issue (R. Miller, personal observation). In total 39 independent observations (experimental units) with greater than 65% mortality at the time of harvest were removed from the original dataset of 640 observations. Data were also inspected for extreme moisture content values, which can impact dry matter yield calculations. Two likely sources of error were 1) premature removal of samples from the drying oven, resulting in excessively low moisture content estimates, and 2) extraneous moisture

contamination at harvest during wet conditions, leading to excessively high moisture values. Moisture values that were greater or less than three standard deviations of the mean across all samples were considered outliers and were removed from the dataset. In order to retain a yield value for the particular observation, the mean of the remaining three replicates was applied to the plot fresh weight of the outlying moisture content. This was done for 15 samples in total from four of nine trials, which represented 2.3% of the total observations considered in this analysis. For the Boisbriand, QC trial, a single wood sample was collected for each cultivar and the moisture content was applied to all four replicates of that cultivar. The mean moisture content of these samples was reported at 32.7% (SD 2.90%), and it was assumed that all samples had not dried sufficiently, considering the overall mean moisture content across all other trials was 46.9% (SD 4.77%). A correction factor equaling the difference between these two mean values was added to each of the observations originally reported for the Boisbriand trial. This value was 14.2 %, which was also very close to the value of 3 standard deviations of the overall mean (14.3%). After accounting for survival and moisture content adjustments, 601 observations were available for analysis from the original 640 from 16 GEN in 10 ENV.

2.3.4 Statistical analysis

We chose to analyze G×E interactions in our first-rotation yield dataset using the Additive Main effects and Multiplicative Interactions (AMMI) model. The AMMI model is a combination of Analysis of Variance (ANOVA) and Principal Components Analysis (PCA), where the G×E interaction, contained in the residual of the additive GEN and ENV main effects model, is subjected to PCA and the interaction sum of squares (SS) are partitioned into a series of interaction principal component (IPC) axes, were IPC1 is the first interaction PC axis, and so on. The AMMI0 model indicates that no IPC axes are included and the model is equivalent to the

additive (main effects only) ANOVA. AMMI1 includes the first IPC axis, and so on, while AMMIF is the full model that includes all axes and is equal to the raw data. The maximum number of IPC axes for a given dataset is one less the minimum number of GEN or ENV (Gauch, 1992, p. 85), but typically the majority of the interaction signal is captured in the first few IPC axes, with later IPC axes containing increasing amounts of interaction noise, and decreasing amounts of signal (Gauch, 2012). Therefore, a more parsimonious model containing a lower number of axes is favored and the remaining axes are relegated to a pooled residual. The statistical significance of each axis is often assessed with an *F*-test where the degrees of freedom (DF) for each axis are calculated according to Gollob (1968) (see also Gauch, 1988). More recently, Piepho (1995) demonstrated that the traditional *F*-test can be too liberal and suggested a more conservative F_R-test for determining the significance of each IPC axis. Also, Gauch (1992, p. 147) suggested a simple test for model diagnosis and selection, which involves estimation of the G×E noise SS by multiplying the G×E DF by the mean square error. Consequently the signal G×E SS can be estimated by subtracting the G×E noise SS from the G×E total SS. Therefore, IPC axes can be assessed by the amount of G×E signal SS that are recovered, instead of the total G×E SS. Selection of higher order models that include greater numbers of axes must be weighed not only in terms of statistical significance, but also in terms of practicality, because higher-order AMMI models and especially the noisy AMMIF tend to have a large roster of genotypes that win in at least one environment, and hence such models produce an unmanageable number of mega-environments (Gauch, 2013). Parsimonious models are often preferred, and AMMI models are most useful when the multiplicative terms have agricultural interpretability (Gauch, 2013). Equation 1 gives the general form of the AMMI model:

$$Y_{ger} = \mu + \alpha_g + \beta_e + \Sigma_n \lambda_n \gamma_{gn} \delta_{en} + \rho_{ge} + \varepsilon_{ger} \qquad (1)$$

where Y_{ger} is the yield of the g^{th} genotype in the e^{th} environment for the r^{th} replicate, μ is the grand mean, α_g is the genotype *g* mean deviation (genotype mean minus grand mean), β_e is the environment *e* mean deviation, λ_n is the singular value for n^{th} IPCA axis, γ_{gn} is the genotype *g* eigenvector value for IPCA axis *n*, δ_{en} is the environment *e* eigenvector value for IPCA axis *n*, ρ_{ge} is the residual, and ε_{ger} is the experimental error.

The AMMI analysis was performed using MATMODEL V3.0, an open source statistical program designed specifically for the analysis of G×E interactions (Gauch & Furnas, 1991; Gauch, 2007). MATMODEL performs the combined ANOVA/PCA analysis and also delineates mega-environments, which allows for the exploration of specific adaptation to particular edaphic or climatic conditions (Gauch & Zobel, 1997).

MATMODEL does not analyze the experimental design, hence we first analyzed the experimental design using PROC MIXED in SAS® version 9.4 (SAS Institute Inc., 2013), with block nested within ENV, and coded as a random effect. The main effects of GEN and ENV and the G×E interaction term were considered fixed effects. The TYPE3 option in PROC MIXED was invoked in order to obtain expected SS for the fixed effects. Tukey's studentized range (HSD) post-hoc test was performed for means separation among GEN. The least squared means from the SAS output were then supplied to MATMODEL to perform the PCA of the interaction and for the mega-environment analysis. The random error variance from the SAS PROC MIXED output was used to calculate the F-tests in the AMMI analysis. Finally, the environment main effects and IPC scores were used in correlation analyses with the various environmental variables using PROC CORR in SAS® version 9.4.

2.4 Results

2.4.1 ANOVA and genotype main effects

The grand mean for first rotation yields for this base case dataset based on the mixed model analysis of variance was 6.96 Mg ha^{-1} yr^{-1}. The main effects of ENV and GEN and the G×E interaction were all highly significant ($p < 0.0001$; Table 2.3), and accounted for 82, 6 and 12% of treatment SS, respectively. The blocking effect (nested within ENV) accounted for a relatively large amount of random error variance (Table 2.3).

Table 2.3 Mixed model analysis of variance for first rotation yields of 16 shrub willow genotypes in 10 environments showing F-test results for fixed effects and variance components for random effects.

Source		DF	SS	MS	*F* Value	Pr > *F*
Fixed	ENV	9	5247.57	583.06	28.01	<0.0001
	GEN	15	381.03	25.40	9.19	<0.0001
	G×E	135	765.39	5.67	2.05	<0.0001
		DF	VC	Pct		
Random	BLK(ENV)	30	1.27	31.43		
	Error	411	2.76	68.57		

DF, degrees of freedom; SS, sum of squares; MS, mean square; ENV, environment; GEN, genotype; BLK, block; VC, variance component; Pct, percent of total variance

Although the main effect of genotype accounted for only 6% of the treatment SS, it contains important information about patterns of broad adaptation. The cultivar *Salix viminalis* × *S. miyabeana* 'Fabius', a triploid hybrid in diversity group 8 (DG8) was the overall greatest yielding GEN across the 10 ENV tested, with a mean yield of 8.58 Mg ha^{-1} yr^{-1} (Table 2.4). Two other *Salix viminalis* × *S. miyabeana* triploid hybrids in DG8, 'Otisco' and 'Tully Champion', ranked 2nd and 4th in overall yield, respectively (Table 2.4, Figure. 2.1a). Two of the top five

GEN were tetraploid *S. miyabeana* (DG5), including ‘Canastota’ a progeny selected from a cross between ‘SX61’ and ‘SX64’. Two triploid *S. purpurea* × *S. miyabeana* cultivars from DG9, ‘Oneida’ and ‘Millbrook’, were ranked 6th and 7th overall, respectively (Figure 2.1a). Triploids showed the greatest relative yields (GEN yield / ENV mean yield averaged over all ENV), with the exception of two cultivars, ‘Truxton’ and ‘Owasco’, which performed at or below ENV means (Figure 2.1b). The cultivars *S. miyabeana* ‘SX61’, ‘SX64’ and *S.* × *dasyclados* ‘SV1’ were cultivars that showed promise in earlier trials, and subsequently were used as check cultivars in this yield trial network. The top two improved cultivars, ‘Fabius’ and ‘Otisco’, performed better than all three check cultivars and four other improved cultivars performed better than the check clone mean yield of 7.14 Mg ha^{-1} yr^{-1}. The GEN with the lowest mean yields were diploid *S. eriocephala* ‘S25’ (DG4), a North American native willow species, and two hybrid cultivars of *S. koriyanagi* × *S. purpurea* ‘Onondaga’ and ‘Allegany’ (DG6b). ENV mean yields ranged from 2.57 to 11.3 Mg ha^{-1} yr^{-1}, with the greatest yields in eastern US and Canada, and the lowest yields occurring in the Upper Peninsula of MI, USA and Saskatoon, SK, CA.

Table 2.4 Adjusted mean first rotation shrub willow yields (Mg ha^{-1} yr^{-1}) for 16 genotypes in 10 environments in North America.

	Environments*										
Epithet	Midd	Bois	Stor	Wase	Fred	Cons	Esca	Sask	Skan	Brim	MEAN
'Fabius'	13.96	13.17	13.27	10.01	8.19	8.76	8.83	4.38	1.95	3.25	8.58
'Otisco'	11.95	12.39	11.10	7.66	7.54	6.55	9.38	4.46	3.18	2.91	7.71
'SX64'†	11.11	13.61	9.15	10.13	8.01	8.61	5.94	3.34	3.21	3.08	7.62
'Tully Champion'	13.34	13.15	6.65	7.57	8.22	5.61	7.51	5.80	3.75	3.70	7.53
'Canastota'	14.56	13.36	8.78	8.98	7.71	7.75	5.20	3.53	2.29	2.47	7.46
'Oneida'	10.63	8.85	12.31	9.80	6.50	8.89	6.91	4.67	3.25	2.41	7.42
'Millbrook'	10.77	10.04	9.23	9.11	8.00	8.11	7.96	4.13	2.24	2.63	7.22
'SX61'	11.97	10.45	9.44	9.62	6.78	7.05	7.89	2.75	3.05	3.14	7.21
'Fish Creek'	11.18	10.78	9.77	9.07	7.59	8.17	6.30	4.03	2.00	1.74	7.06
'Truxton'	12.32	9.95	9.84	8.96	8.32	6.59	5.71	3.60	3.40	1.74	7.04
'Sherburne'	11.92	10.72	9.82	8.61	6.53	7.02	5.50	3.32	1.89	2.30	6.76
'SV1'	10.92	11.54	6.03	4.11	7.62	7.81	9.02	3.28	2.47	3.23	6.60
'Owasco'	10.13	8.75	7.18	8.85	8.29	6.32	6.44	4.20	3.15	2.56	6.59
'Allegany'	8.31	9.31	6.88	7.51	5.26	6.32	6.94	3.10	2.21	2.13	5.80
'Onondaga'	8.36	7.54	5.54	7.34	5.10	6.84	6.67	3.60	2.06	2.02	5.51
'S25'	9.44	8.52	5.39	6.60	6.52	3.87	3.90	3.81	2.39	1.74	5.22
MEAN	11.30	10.76	8.77	8.37	7.26	7.14	6.88	3.87	2.66	2.57	

*Environment names are truncated to the first four letters, with full environment names given in Table 2.2.
†Check cultivars underlined.

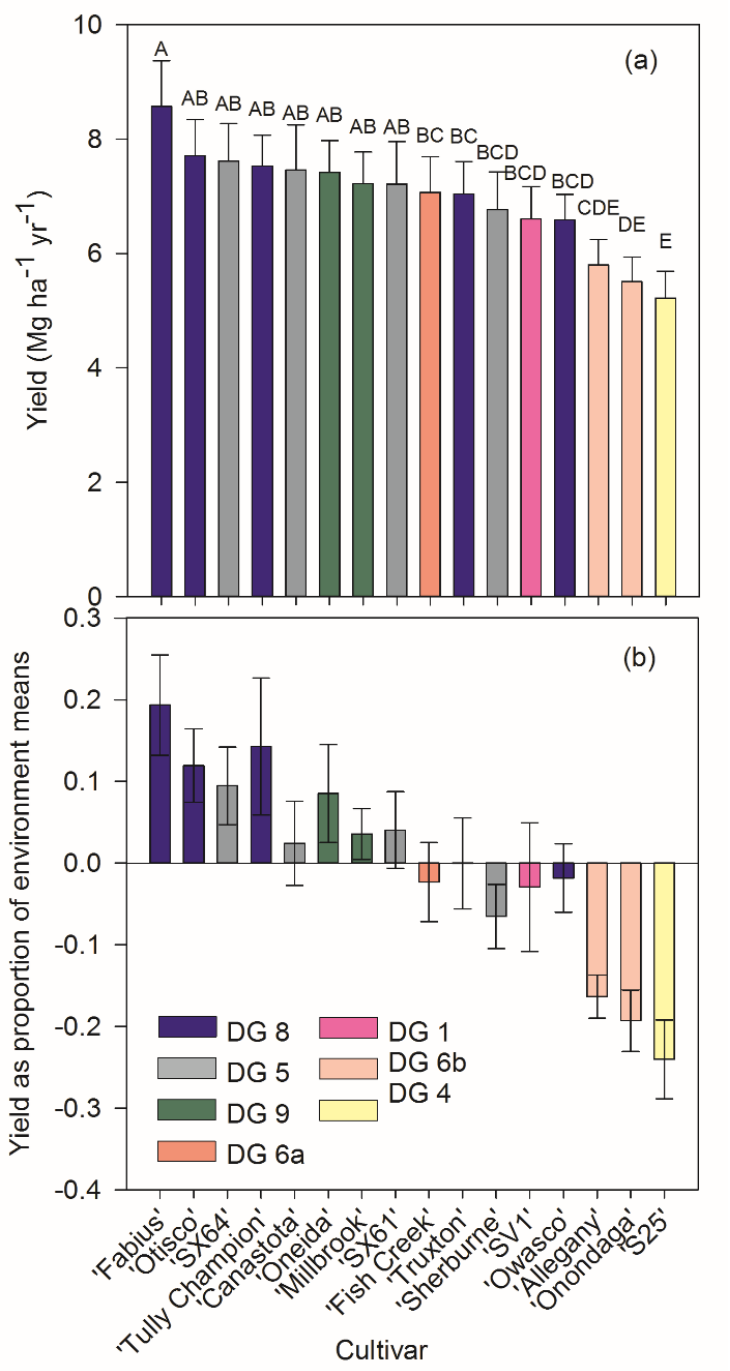


Figure 2.1 First rotation shrub willow mean yields (a) and yields as a proportion of environment means (b) for 16 genotypes in 10 environments in North America. Bars are shaded according to the diversity groups (pedigrees) presented in Table 2.1. Bars in (a) with different letters indicate significant ($p < 0.05$) differences according to Tukey's studentized range test (HSD). Error bars in (a) are ± one standard error of the mean. Proportions in (b) were calculated as the yield of a genotype divided by the environment mean, averaged across the 10 environments.

2.4.2 AMMI analysis

The AMMI2 model ANOVA revealed that the main effects of GEN and ENV plus the first two IPC axes accounted for 95.5% of the treatment SS with only 68 (out of 159) treatment DF (Table 2.5). Using the error variance obtained from the SAS PROC MIXED output (Table 2.3) both the F-tests and the more conservative F_R-tests proved IPC1 and IPC2 to be significant ($p < 0.0002$); however, all subsequent axes were not significant and were therefore pooled into a discarded residual. The G×E interaction term is inherently a mixture of true signal in the data (i.e., systematic rank changes amount GEN) and noise. The amount of G×E noise can be estimated by multiplying the G×E DF by the mean square error (Gauch, 2013). Using the mean square error variance obtained from the SAS PROC MIXED output (Table 2.3), the G×E noise SS for this dataset was estimated to be 373 (46%), while the G×E signal accounts for 438.5 (54%) of the total G×E SS. IPC1 captured 291 SS, or 66.4% of the G×E signal SS. The cumulative SS accounted for by the first two IPC axes is 507, which is greater than the estimated G×E signal SS, suggesting that IPC2 contains a combination of mostly signal and some noise. Therefore AMMI1 does a reasonable job of capturing the majority of the G×E signal in a parsimonious model. However, in terms of accurately describing our data, AMMI2 might be slightly better, although the more important consideration is that AMMI1 (and AMMI2) is considerably more accurate than the raw data AMMIF since its additional IPC3 and higher components (which are combined in the residual in Table 2.5) capture a SS of 304, which is mostly noise.

Table 2.5 Additive Main effects and Multiplicative Interactions (AMMI) analysis of variance for first rotation yields of 16 shrub willow genotypes in 10 environments showing the first two IPC axes.

Source	DF	SS	MS	*F* Value*	Pr > *F*	F_R Value	Pr > F_R
TRT	159	6774.19	42.60	15.42	<0.0001		
GEN	15	454.50	30.30	10.97	<0.0001		
ENV	9	5508.20	612.02	221.51	<0.0001		
G × E	135	811.49	6.01	2.18	<0.0001		
IPC1	23	290.96	12.65	4.58	<0.0001	2.18	<0.0001
IPC2	21	216.04	10.29	3.72	<0.0001	1.68	<0.0002
Residual	91	304.49	3.35	1.21	0.99	1.21	0.11

DF, degrees of freedom; SS, sum of squares; MS, mean square; TRT, treatment; ENV, environment; GEN, genotype; BLK, block.

*The error mean square from Table 3 was used in calculations of *F* and F_R values.

A useful tool for interpreting the AMMI analysis is the AMMI1 biplot, where the additive main effects of GEN and ENV are plotted on the x-axis in units of yield (Mg ha^{-1} yr^{-1}), and the IPC1 scores are plotted on the y-axis, which have units expressed as the square root of the yield (Gauch, 1992, p. 85). By representing both the main effects and the majority of the interaction signal in one projection, the AMMI1 biplot captures 92.3% of the treatment SS, and the relationships between main effects and interaction can be observed simultaneously (Figure 2.2). The vertical reference line represents the grand mean yield of the dataset and the horizontal line is placed at zero for the IPC scores, where points farther away from this line indicate GEN or ENV with larger interactions. Genotypes that occur close to the horizontal line can be regarded as having relatively stable yields across ENV. Genotypes and ENV having the same sign for their IPC1 scores have positive interactions, whereas opposite signs give negative interactions. For instance, ‘Fabius’ was the highest yielding GEN and consequently it is farthest along the right side of the x-axis. It also had one of the largest IPC1 scores, generating a positive

interaction in Storrs, CT, but a negative interaction in Skandia, MI. Indeed, the lowest yield for 'Fabius' of 1.95 Mg ha^{-1} yr^{-1} occurred at that location, in comparison with Storrs, CT, where 'Fabius' yielded 13.27 Mg ha^{-1} yr^{-1} (Table 2.4). The IPC1 scores for 'SV1' and 'Tully Champion' are similar and were the most extreme negative scores out of the 16 GEN, so their interaction patterns are the opposite of 'Fabius'. However, 'Tully Champion' has a substantially greater mean yield compared to 'SV1', and it was the top yielding cultivar in many low-yielding ENV (Table 2.4).

When IPC2 is significant (Table 2.5), the AMMI2 biplot can be used to investigate the interaction pattern in IPC1 and IPC2 together (Figure 2.3). The reference lines in Figure 2.3 are drawn through zero for both axes. The crossing of these two lines in the middle of the graph indicates no interaction, and, therefore, a GEN close to this point would be characterized as having stable yields across ENV, and for this dataset the triploid 'Otisco' and the tetraploids 'SX61' and 'SX64' are closest to the origin. 'Fabius' and 'Tully Champion are on the opposite ends of IPC1 scores, but have very similar IPC2 scores. In contrast, 'Onondaga' and 'Canastota' were on the opposite extremes of IPC2 scores, with 'Canastota' having a strong positive interaction with Boisbriand, QC in terms of IPC2, since their IPC scores are of the same sign. Unlike Figure 2.2, there is no information about main effects in Figure 3, only interactions, but it is useful for understanding the relationships between AMMI1 and AMMI2.

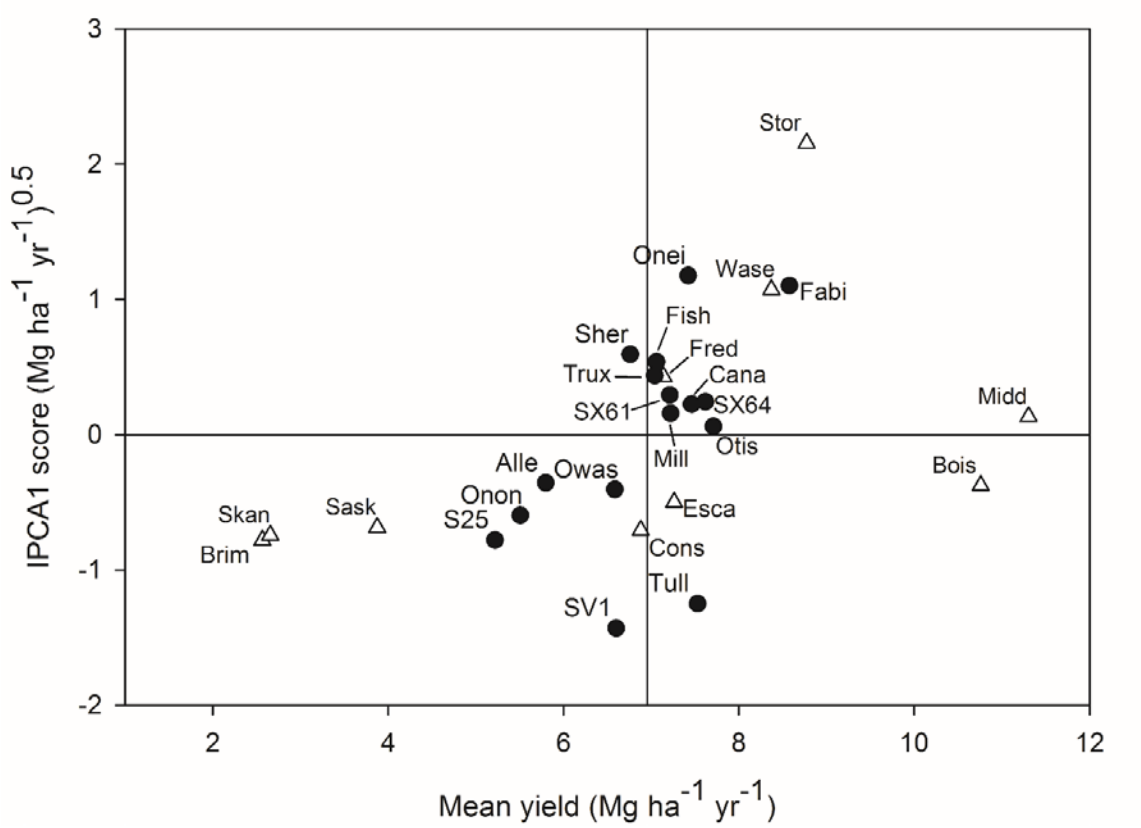


Figure 2.2 AMMI1 biplot for the shrub willow yield trial network. Genotype (●) and environment (△) means are on the x-axis, and the IPC1 scores are shown on the y-axis. Genotype and environment names are truncated to the first four letters and full names can be found in Table 2.1 and Table 2.2, respectively. The vertical reference line represents the grand mean, while the horizontal line crosses at an IPC score of zero.

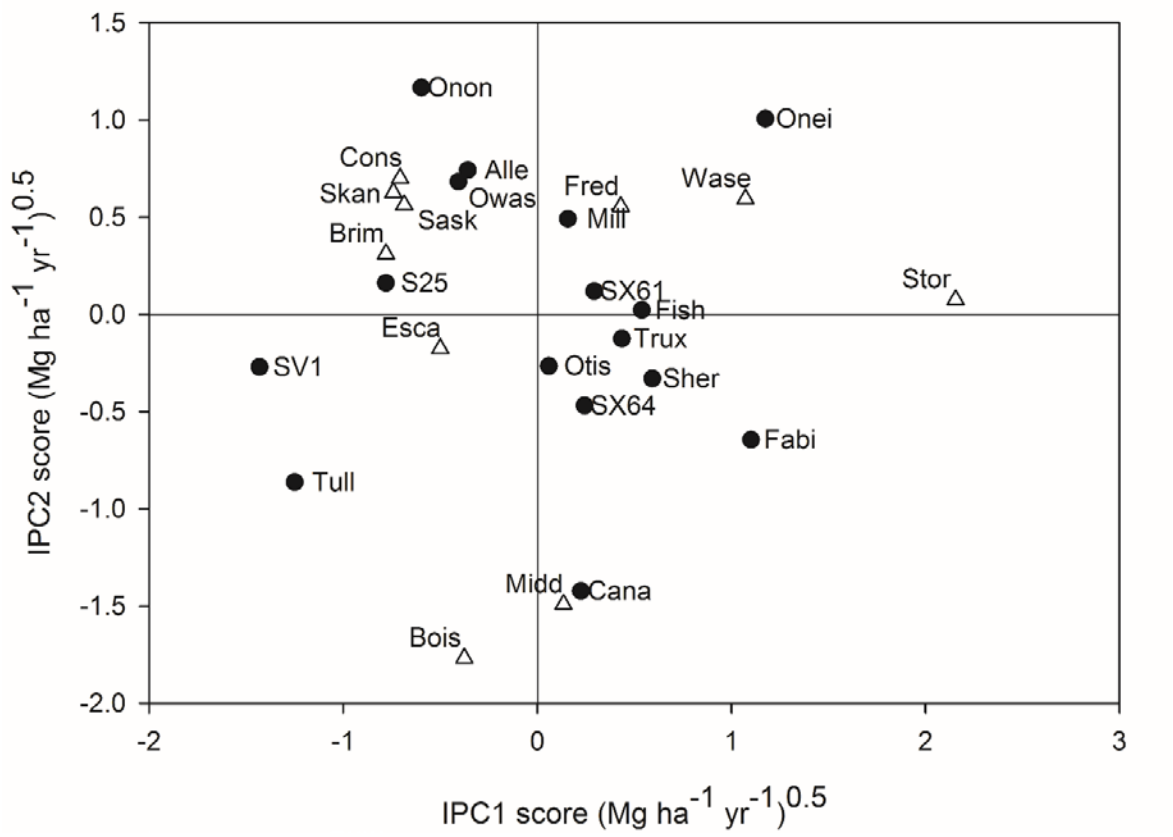


Figure 2.3 AMMI2 biplot for the shrub willow yield trial network. Genotype (●) and environment (△) IPC1 scores are shown on the x-axis, and IPC2 scores are on the y-axis. Genotype and environment names are truncated to the first four letters and full names can be found in Table 2.1 and Table 2.2, respectively. The vertical and horizontal reference lines are drawn through zero for both axes.

2.4.3 Mega-environment delineations

Another useful graphical representation of AMMI1 analysis is a plot of the AMMI1 nominal yields (AMMI1 model yields for each GEN, without the ENV deviation, see equation 1), against the ENV IPC1 scores (Gauch & Zobel, 1997). Figure 4 shows nominal yields for the 16 GEN across the 10 ENV plotted as straight lines and the vertical positions of the lines relative to one another at a given ENV IPC1 score indicate the yield rankings. This plot is very useful for visualizing GEN cross-overs, and for ease of interpretation only the most relevant GEN have been highlighted and their names provided. For instance, ‘Fabius’ had the greatest yields in five

ENV ranging in IPC1 scores from -0.38 to 2.16. But for the other five ENV with scores -0.50 and lower, a switch point occurs where ‘Tully Champion’ outperforms ‘Fabius’ according to the AMMI1 model predicted yields. This situation illustrates the concept of narrow adaptation, where GEN perform similarly in a group of ENV with similar characteristics.

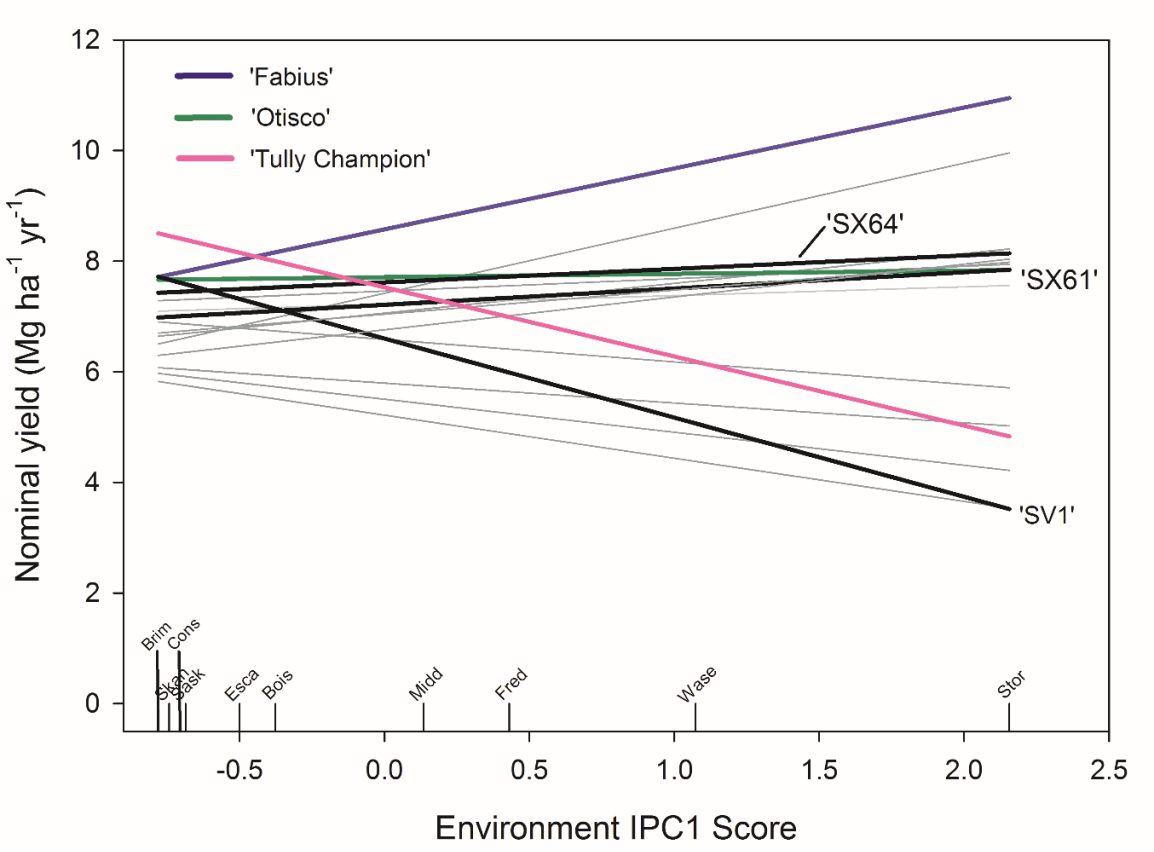


Figure 2.4 Shrub willow genotypic responses according to the AMMI1 model. The 10 environment IPC1 scores are represented on the x-axis, while the genotype nominal yields (AMMI1 model yields without the environment deviations) are shown on the y-axis. All 16 genotypes are represented individually by a straight line. Only relevant genotypes have been labeled. Check cultivars are solid black lines. Environment names are truncated to the first four letters and full names can be found Table 2.2.

The degree of environmental sensitivity of a GEN can also be inferred by observing the slopes in Figure 4. ‘Fabius’ and ‘Tully Champion’ have steep, but opposite slopes due to their opposite interactions. ‘Otisco’ was ranked 2nd in overall yield and was relatively insensitive to

ENV, as evidenced by its near zero slope (Figure 2.4). Also, similarities among GEN can be observed where slopes are nearly parallel. The line just below 'Fabius' on the right side of Figure 4 is that of 'Oneida', which is similar to 'Fabius', suggesting that they reacted to ENV similarly. However, the mean yields of 'Fabius' were considerably greater than 'Oneida', making 'Fabius' an obvious superior choice. Similarly 'SV1' had an interaction pattern that mirrored 'Tully Champion', but again, the predicted AMMI1 yields were lower for 'SV1'.

AMMI can also be used to delineate mega-environments, which are defined as subdivisions of a crop's potential growing range that share similar genotype winners, presumably due to similar biotic and abiotic stresses, but that are not necessarily contiguous (Gauch & Zobel, 1997; Yan et al., 2000). Subdivision of a crop's growing region can help to improve yields by targeting GEN with specific adaptation to ENV where they are likely to perform best (Gauch & Zobel, 1997), especially if there are identifiable environmental or biological patterns associated with the IPC analysis that can be extended to ENV beyond those where crop yields have been tested (Gauch, 2013). MATMODEL uses key switch points in nominal yields among winning GEN across IPC1 scores to delineate mega-environments. For this shrub willow yield dataset, the main switch point for the AMMI1 model is clearly illustrated in Figure 4, where 'Fabius' and 'Tully Champion' switch between first and second rank at a value of about -0.44 along the ENV IPC1 range. Therefore, AMMI1 defines two winners which happen to divide the 10 ENV equally between them, where 'Fabius' is declared the overall winner, but 'Tully Champion' is superior in the lower yielding ENV.

Since the AMMI analysis consists of a family of models, with higher order models incorporating greater numbers of interaction components, higher order models generally result in an increased number of mega-environments and winners (Gauch, 2013). To illustrate this point,

Table 2.6 shows the top five rankings for three AMMI model family members. The ENV are listed by IPC1 scores. AMMI1 divides the growing region into two mega-environments as described above, with only two first place winners, ‘Fabius’ and ‘Tully Champion’. ‘Fabius’ takes second place in most ENV where ‘Tully Champion’ was ranked first. ‘Oneida’ ranks behind ‘Fabius’ in more favorable ENV, while ‘Otisco’ ranks third in many of the less favorable ENV. The main differences between AMMI1 and AMMI2 are that Boisbriand, QC is declared as a separate mega-environment with ‘Canastota’ the top ranking GEN, and to some degree ‘Fabius’ is relegated to lower ranks in the less favorable ENV. The AMMIF model, which is equivalent to the raw data, is also included for comparisons. Should the full data be accepted as most accurate, it would result in a very complex array of mega-environments with seven different cultivars being declared winners in seven narrowly defined sub-regions. AMMI1 with its simplified mega-environment winner pattern is likely best for making cultivar selections far less complicated. AMMI2 is likely the most accurate choice for modeling overall genotypic performance in this dataset, but the inclusion of one winner in one ENV complicates recommendations and justification for its inclusion would likely need to be verified with more testing.

Table 2.6 Rankings of the top five genotypes for in 10 environments (ENV) based on three AMMI models. AMMI1 includes only the first IPC axis and AMMI2 includes both the first and second IPC axes which were both significant based on the F_R-test. AMMIF represents the raw data.

	AMMI1 Rank					AMM2 Rank					AMMF Rank				
ENV*	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Stor	Fabi†	Onei	Fish	SX64	Sher	Fabi	Onei	Fish	SX64	Sher	Fabi	Onei	Otis	Trux	Sher
Wase	Fabi	Onei	SX64	Otis	Cana	Fabi	Onei	Mill	Fish	Otis	SX64	Fabi	Onei	SX61	Mill
Fred	Fabi	Onei	Otis	SX64	Cana	Fabi	Onei	Otis	Mill	SX64	Onei	Fabi	SX64	Fish	Mill
Midd	Fabi	Otis	SX64	Onei	Cana	Fabi	Cana	*Tull*	SX64	Otis	Cana	Fabi	*Tull*	Trux	SX61
Bois	Fabi	*Tull*	Otis	SX64	Cana	Cana	*Tull*	Fabi	SX64	Otis	SX64	Cana	Fabi	*Tull*	Otis
Esca	*Tull*	Fabi	Otis	SX64	Cana	*Tull*	Fabi	Otis	Cana	SX64	Trux	Owas	*Tull*	Fabi	SX64
Sask	*Tull*	Fabi	Otis	SV1	SX64	*Tull*	Otis	Fabi	SV1	Mill	*Tull*	Onei	Otis	Fabi	Owas
Cons	*Tull*	Fabi	Otis	SV1	SX64	*Tull*	Otis	Mill	SV1	Owas	Otis	SV1	Fabi	Mill	SX61
Skan	*Tull*	Fabi	Otis	SV1	SX64	*Tull*	Otis	SV1	Mill	Fabi	*Tull*	Trux	Onei	SX64	Otis
Brim	*Tull*	SV1	Fabi	Otis	SX64	*Tull*	SV1	Otis	Fabi	SX64	*Tull*	Fabi	SV1	SX61	SX64

*ENV, Environment. Names are truncated to the first four letters. Full names can be found in Table 2.2.

†Genotype names are truncated to four letters, with full names presented in Table 2.1. The overall top-yielding cultivar, 'Fabius' is underlined throughout the table, and the second mega-environment winner from AMMI1, 'Tully Champion' is italicized throughout the table.

2.4.4 Edaphic and climatic variables

The AMMI analysis of the G×E interaction operates solely on yield data, with an implicit interpretation that general patterns in yields are a reflection of underlying environmental characteristics of test locations. However, it may be desirable to associate yield patterns with known environmental characteristics, and IPC scores can be used in simple correlations when environmental variables for test locations are available (Van Eeuwijk, 1995). ENV yield was significantly ($p < 0.05$) correlated with mean annual growing degree days and marginally significantly ($p < 0.10$) associated with longitude and growing season precipitation (Table 2.7). ENV IPC1 scores were also significantly correlated with growing degree days, mean annual minimum temperatures and latitude (Table 2.7), which would covary with each other, but suggest a strong relationship between interaction patterns and temperature. IPC2 scores were positively correlated with elevation (Table 2.7).

Table 2.7 Pearson product-moment correlation coefficients between mean yields (Mg ha^{-1} yr^{-1}), IPC scores and edaphic and climatic variables for the 10 environments in the yield trial network.

Variable	Yield	IPC1	IPC2
Latitude	-0.534	-0.619*	0.098
Longitude	0.587*	0.300	-0.503
Elevation (m)	-0.496	-0.204	0.785**
Mean annual precipitation (mm)	0.527	0.346	-0.221
Mean April-October precipitation (mm)	0.578*	0.525	-0.195
Mean annual growing degree days (base 10°C)	0.650**	0.827**	-0.287
Mean annual minimum temperature (°C)	0.394	0.729**	-0.166
Mean annual solar radiation (MJ m^{-1} day^{-1})	-0.038	0.479	0.238
Soil organic matter (%)	0.261	-0.116	-0.013
Soil pH	0.028	-0.191	-0.320
Depth to water table (cm)	-0.131	-0.300	0.009

*$p < 0.10$; **$p < 0.05$

2.4.5 Expanded datasets and analyses

As noted earlier, our yield trial network contained more GEN and test ENV than those included in the above AMMI analysis (601 observations out of ~1530 available). However, the MATMODEL program allows for imputation of missing treatment cells in the two-way table of means using the Expectation-Maximization (EM) algorithm (Gauch & Zobel, 1990). In order to more fully explore the yield database, two additional datasets were assembled that expanded the original complete set of 16 GEN in 10 ENV. The first dataset had an additional 6 GEN that included 10 missing treatments (4.5%) out of a possible 220, for a total of 22 GEN in 10 ENV. The second dataset added 6 ENV with 15 missing treatments (5.9%) out of 256. It has recently been shown that using the EM method for data imputation with missing data proportions of 5-

10% do not significantly reduce the predictive ability of the AMMI model, especially when a small number of IPC axes (one or two) are chosen (Rodrigues et al., 2011; Paderewski & Rodrigues, 2014). The ANOVA tables and mega-environment winners for the two expanded datasets are provided in Table 8. The ANOVA for the two expanded datasets show greater overall variance in the treatments, as expected, but the G×E signal SS were similar at 38.7% for the increased GEN and 36.7% for the increased ENV scenarios (Table 2.8). The IPC1 axes accounted for 82.4 and 76% of the G×E signal SS for the expanded GEN and expanded ENV scenarios, respectively. Interestingly, the mega-environment analyses remain remarkably similar to those of the base case scenario (Table 2.9). Even with six additional GEN, 'Fabius' remains the winner in six ENV and 'Tully Champion' is the winner in four of the lowest yielding ENV. When six ENV were added for a total of 16, 'Fabius' wins in seven, and 'Tully Champion' in eight (Table 2.9). 'SV1' wins in one environment, Belleville, NY, but 'Tully Champion' is a close second, and often combining a small mega-environment with a larger one occupied by a close winner is justified (Gauch, 2013). The addition of these two scenarios reinforces the superiority of 'Fabius' and 'Tully Champion' relative to the cultivars tested in the analysis.

Incidentally, MATMODEL can also provide the Finlay-Wilkinson joint regressions analysis (Zobel et al., 1988; Gauch, 2007), which we applied to the original dataset of 16 GEN in 10 ENV. The joint regression analysis only accounted for 23.2% of the G×E SS, compared to 35.9% for AMMI IPC1 (Table 2.10). Based on GEN slopes, the joint regression analysis of the GEN with the best combination of stable slopes and greater yields would be as 'Otisco' and 'SX64', with the high yields and slopes slightly above 1, and 'Tully Champion' and 'Oneida' with somewhat lower yields and slopes slightly less than 1 (Table 2.10). These results are somewhat in agreement with AMMI, but selection based on stability would likely rule out the

potential gains from including ‘Fabius’ in optimal locations.

Table 2.8 Additive Main effects and Multiplicative Interactions (AMMI) ANOVA tables including the first IPC axis for six additional genotypes and six additional environments

22 genotypes in 10 environments (Grand mean = 6.813 Mg ha^{-1} yr^{-1})					16 genotypes in 16 environments (Grand mean = 6.544 Mg ha^{-1} yr^{-1})				
Source	DF	SS	MS	Pr > *F*	Source	DF	SS	MS	Pr > *F*
Total	829	11833.78	14.27		Total	952	14360.77	15.08	
TRT	219	9532.35	43.53	<0.0001	TRT	255	11884.29	46.61	<0.0001
GEN	21	611.80	29.13	<0.0001	GEN	15	581.24	38.75	<0.0001
ENV	9	7757.37	861.93	<0.0001	ENV	15	10039.24	669.28	<0.0001
G×E	189	1163.18	6.15	<0.0001	G×E	225	1263.81	5.62	<0.0001
IPCA1	29	371.07	12.80	<0.0001	IPCA1	29	352.93	12.17	<0.0001
Residual	160	792.11	4.95	0.012	Residual	196	910.88	4.65	0.008
Error	610	2301.43	3.77		Error	697	2476.48	3.55	
G×E Signal		450.11	38.70%		G×E Signal		464.37	36.74%	
G×E Noise		713.07	61.30%		G×E Noise		799.44	63.26%	
G×E Total		1163.18			G×E Total		1263.81		
IPC1		371.07	82.44%		IPC1		352.93	76.00%	

Table 2.9 Additive Main effects and Multiplicative Interactions (AMMI) mega-environment analysis for 6 additional genotypes and 6 additional environments.

22 genotypes in 10 environments									
	AMMI model								
Genotype*	0	1	2	3	4	5	6	7	F
'Fabius'	10	6	3	2	3	1	1	1	1
'Oneida'			2	2	1	1	1		
'Canastota'					1	2	1	1	1
'Truxton'								1	1
'Oneonta'								1	1
'Otisco'							1		
'SX64'							1	2	2
'Saratoga'						1	1	1	1
'Tully Champion'		4	5	5	4	4	4	3	3
'SV1'				1	1	1			
Mega-environment Count	1	2	3	4	5	6	7	7	7

*Genotypes are listed in IPC1 order; 12 genotypes are not listed because they never win

16 genotypes in 16 environments									
	AMMI model								
Genotype*	0	1	2	3	4	5	6	7	F
'Fabius'	16	7	5	6	5	3	3	3	2
'Canastota'						2	2	2	2
'SX64'							1	2	2
'Otisco'							2	2	2
'Oneida'			1		1	1			1
'Truxton'									1
'Millbrook'									1
'Tully Champion'		8	8	7	6	6	5	5	3
'Fish Creek'			2	1	3	3	3	2	2
'SV1'		1		2	1	1			
Mega-environment Count	1	3	4	4	5	6	6	6	9

*Genotypes are listed in IPC1 order; six genotypes are not listed because they never win

Table 2.10 Joint regression model analysis for 16 shrub willow genotypes in 10 environments.

Source	DF	SS	MS	Pr > *F*
Total	600	8537.53	14.23	
TRT	159	6760.66	42.52	<0.0001
GEN	15	451.89	30.13	<0.0001
ENV	9	5492.19	610.24	<0.0001
G x E	135	816.58	6.05	0.0012
Joint Regression	1	136.74	136.74	<0.0001
GEN Regressions	14	59.64	4.26	0.395
ENV Regressions	8	47.80	5.98	0.161
Residual	112	572.40	5.11	0.049
Error	441	1776.87	4.03	

Linear regression model parameters

Genotype	Mean	Slope	R^2
'Fabius'	8.60	0.364	0.554
'Canastota'	7.51	0.338	0.553
'Sherburne'	6.87	0.128	0.478
'SX64'	7.62	0.125	0.113
'Fish Creek'	7.10	0.085	0.162
'Otisco'	7.71	0.079	0.051
'Truxton'	7.04	0.066	0.050
'SX61'	7.21	0.045	0.029
'Millbrook'	7.22	-0.022	0.012
'Tully Champion'	7.53	-0.033	0.004
'Oneida'	7.42	-0.064	0.017
'SV1'	6.62	-0.123	0.041
'Allegany'	5.79	-0.202	0.481
'Owasco'	6.60	-0.219	0.434
'S25'	5.22	-0.242	0.389
'Onondaga'	5.55	-0.325	0.560
Environment	**Mean**	**Slope**	**R^2**
Storrs, CT	8.83	1.236	0.367
Boisbriand, QC	10.76	0.704	0.194
Middlebury, VT	11.31	0.665	0.236
Waseca, MN	8.37	-0.002	0.000
Fredonia, NY	7.18	-0.132	0.014
Constableville, NY	6.97	-0.147	0.010
Escanaba, MI	7.21	-0.191	0.043
Brimley, MI	2.60	-0.591	0.516
Saskatoon, SK	3.89	-0.720	0.444
Skandia, MI	2.66	-0.821	0.594

2.5 Discussion

Our analysis of this North American shrub willow yield trial network data has demonstrated the importance of G×E interactions in short rotation woody crop productivity. Our evaluation of first rotation yields using the AMMI model has accomplished the main objectives of the study, which were to identify GEN with broad adaption, define sub-regions where particular GEN exhibit specific adaption, and to identify environmental variables that help to explain patterns in yield. Despite the proven success of AMMI in accurately diagnosing G×E interactions from a range of agronomic and horticultural crops, we have not seen any other published reports of its use in short rotation coppice research. AMMI helped to resolve signal from noise and we selected the more parsimonious model, AMMI1, as the best representation of the data, simplifying the mega-environment analysis with just two winning GEN, 'Fabius' and 'Tully Champion', as opposed to the raw data with seven of the 16 GEN in our dataset winning in at least one environment (Table 2.6). In further support of the AMMI1 analysis winners, inclusion of additional GEN or additional test locations did not change the mega-environment analysis outcomes for AMMI1 (Table 2.8).

'Fabius' was the top-yielding cultivar with an overall mean of 8.58 Mg ha^{-1} yr^{-1}, setting a standard for optimal yields and broad adaptability. Also, because of its positive interaction with high yielding ENV, it is well adapted to favorable growing conditions. 'Tully Champion' had an opposite pattern of interaction and appears to be adapted to less desirable growing conditions. 'Tully Champion' and 'SV1' interacted with the test ENV similarly (Figure 2.4), but 'Tully Champion' consistently outperformed 'SV1' make it a much better check cultivar for evaluating future breeding material. Similarly, 'Otisco' and 'SX64' had fairly stable yields, showing little

interaction with environment regardless of quality, but ‘Otisco’ was ranked second in overall mean yield. All three of these superior cultivars (‘Fabius’, ‘Tully Champion’ and ‘Otisco’) are of the triploid pedigree of *S. viminalis* × *S. miyabeana*, and they outperformed the mean of the check cultivars by 5 to 20%, demonstrating that yield gains can be achieved through novel hybridization in shrub willow. This affirms the findings of Serapiglia et al. (2014) for early selections of triploids on a single site, but extends it more broadly across a large set of diverse ENV.

It should be noted that ‘Fabius’ and ‘Tully Champion’ are siblings from the same cross, which makes their divergent patterns in yield across our sites rather remarkable. These two were selected after an evaluation of the family in a replicated trial at a single location in central NY (Smart et al., 2008). There was considerable variability in growth potential across this family and perhaps the single test location was not adequate for detecting differiential response to environmental conditions. The cultivar with the most stable yields in our dataset, ‘Otisco’, shares the same *S. viminalis* mother, ‘SV2’, with ‘Fabius’ and ‘Tully Champion’ (Table 2.1). ‘Owasco’ and ‘Truxton’ are also siblings that belong to the *Salix viminalis* × *S. miyabeana* pedigree (DG8). They share the same father with ‘Otisco’, ‘SX64’, but their overall yields were much lower, suggesting superior combining ability of ‘SV2’ for yield traits. This suggests that interspecific triploid hybridization in *Salix* can confer substantial gains in yield, and future breeding efforts will continue to exploit this potential.

By correlating environment mean yields and IPC scores with edaphic and climatic variables from the test sites, we were able to demonstrate that increased temperatures were positively correlated with both mean yields and IPC1 scores, while the correlation between precipitation and mean yield was marginally significant. We have observed that the triploid

hybrids tend to retain a leaf canopy much later in the season compared to other pedigrees. This could be an indication of lower sensitivity to frost or an ability to exploit solar resources at the tail end of the growing season. Labrecque & Teodorescu (2003) examined yields of two willow species at two contrasting sites in southern Quebec and suggested that growth in that region may be limited by precipitation, but not likely growing season temperatures, based on reported optimal growing conditions in Sweden. Wang & MacFarlane (2012) analyzed poplar (*Populus* spp.) and shrub willow yields of cultivars sourced from Ontario and New York collections at two locations in Michigan and estimated that the difference in growing degree days between northern and southern locations could explain 60% of the variation in yield. In the UK, Aylott et al. (2008) analyzed yields of three willow GEN of differing pedigrees in relation to edaphic and climatic variables from 49 locations. In general, temporal rainfall patterns were found to be the significant factors affecting yields, but the response varied by GEN, with one being particularly sensitive to soil pH. We were unable to find significant correlations with soil pH or organic matter; however, because of the broad geographic range covered in this analysis, perhaps the greatest amount of variability among the test sites was dominated by climatic factors. The subject warrants additional investigation and testing in locations with higher temperatures and moderate rainfall may reveal a greater geographic production range than previously proposed (Walsh et al., 2003).

G×E interactions have been reported elsewhere in recent studies examining willow yields in multiple locations. In eastern Canada, Mosseler et al. (2014a) studied biomass traits of multiple clones from natural accessions of two species, *S. discolor* and *S. eriocephala*, planted on three sites of contrasting fertility. They found no significant rank changes between the two species groups, but large differences in genotypic sensitivities among the GEN within species,

suggesting a high degree of intraspecific genetic variability in adaptation that could be exploited for biomass yield improvement. In Denmark, Larsen et al. (2014) studied yields of Swedish and UK commercial cultivars across five locations and reported significant G×E interactions, but there was one clear winner, 'Tordis' (*S. viminalis* × *S. schwerinii*), with a mean yield of 6.7 Mg ha^{-1} yr^{-1}. In earlier work involving numerous GEN of three willow species across a large number of sites in Sweden, Rönnberg-Wästljung & Thorsén (1988) used a variant of the Finlay-Wilkinson regression to analyze G×E interactions. As in our analysis, linear regressions did not always adequately explain clonal response to ENV. The authors identified stable GEN using regression coefficients and mean yields, but they did not address specific adaptability. In poplar, Zalesny et al. (2009) performed an extensive analysis of 53-79 GEN across four sites and multiple ages in the Upper Midwestern US. They assessed genotypic rankings and segregated GEN based on generalists (consistently high rankings) and specialist (especially variable rankings). More recent attempts to analyze G×E interactions in poplar have incorporated some of the more contemporary statistical techniques used in plant breeding and agronomy. In an analysis of first-year growth of nine GEN in five locations in Spain, Sixto et al. (2011) applied the genotype and genotype x environment (GGE) biplot (Yan et al., 2000), which has some similarities with AMMI biplots and is used to visualize interactions (Gauch, 2006a; Gauch et al., 2008). More recently, Sixto et al. (2014) analyzed biomass production at the end of a three-year rotation for nine GEN in four of the locations from their previous study. They applied mixed model variants of stability parameter models including Finley-Wilkinson and Eberhart-Russell and identified patterns of broad and specific adaptation among GEN and pedigrees. These mixed model approaches often consider ENV as random, and have the advantage of handling unbalanced datasets (Smith et al., 2005).

The importance of developing cultivars with specific adaptation can be emphasized when willow cultivation is placed into broader biological and geographical contexts. In the UK and Swedish breeding programs, improved *S. viminalis* and hybrids such as *S. viminalis* × *S. schwerinii* are highly productive and have demonstrated superior yields in numerous trials compared to other pedigrees (Aylott et al., 2008; Lindegaard et al., 2011; Larsen et al., 2014). In the US, potato leafhopper (*Empoasca fabae* Harris) has proven to be extremely damaging to imported European *S. viminalis* cultivars (Smart & Cameron, 2008). Hybrid crosses of *S. viminalis* with *S. miyabeana* have introduced varying degrees of resistance to potato leafhopper, in addition to improved yields. We have observed more severe damage to 'Tully Champion' compared with 'Fabius' in NY locations (Gouker & Smart, 2015), which may have limited its yield potential on sites of more southern latitude and more eastern longitude. Potentially lower potato leafhopper pressure in northerly ENV may explain the superiority of 'Tully Champion' and 'SV1' at those sites. In Canada, *S. eriocephala* and *S. discolor* have been evaluated as North American native species appropriate for biomass and phytoremediation applications (Mosseler et al., 2014a; Mosseler et al., 2014b). However, testing in the US has shown that natural accessions as well as improved cultivars of *S. eriocephala* can be highly susceptible to leaf rust (*Melampsora* spp) (Cameron et al., 2008; Serapiglia et al., 2013). The improved *S. eriocephala* cultivar 'S25' in our analysis had the lowest overall yield and it is possible that rust infection in combination with deer browse contributed to low performance. In Europe, *Melampsora* rust is a major growth limiting pathogen for multiple willow species and species hybrids (Åhman, 1998; McCracken & Dawson, 2003; Aylott et al., 2008). *Salix purpurea* and hybrid *S. koriyanagi* × *S. purpurea* cultivars are also susceptible to rust, but based on visual observations in the field and the yields of 'Oneida' and 'Millbrook' in this analysis, the *S. purpurea* × *S. miyabeana* diversity

group likely has increased rust resistance. Breeding for rust resistance is an important focus in the US given the European experience.

Inclusion of some ENV in this analysis caused overall yields to be lower compared to other studies, but collectively they provided strong contrasts for discriminating among GEN. In turn, this analysis likely provides a more realistic assessment of yield potentials on marginal lands, given some of the exceptionally low yielding ENV. Since trials are often performed at experiment stations, where growing conditions are optimized or at least less variable, it may seem obvious that many reported yields are often on the high end and they may not be representative (Simmonds, 1991). This becomes even more relevant in the realm of dedicated bioenergy crops, since the premise is that these are purposed for marginal lands; more specifically, where the economic returns are marginal for production of traditional field crops (Stoof et al., 2015), and thus there is reduced competition between food and energy production. Evaluations of shrub willow yield potentials in the US have been largely geographically restricted to regions where institutional knowledge exists, and the true extent of production potential has likely not be adequately tested (Walsh et al., 2003). Given the positive correlations of yield and IPC1 scores with factors relating to increased temperature, perhaps growing willow at lower latitudes will produce greater yields. But genotypic variation in water use efficiency and drought resistance will be important and may help to inform breeding for improved adaptation to warmer climates (Bonosi et al., 2013).

This study has demonstrated the importance of identifying genotypic adaptability for developing cultivar recommendations and guiding future breeding efforts. Genotypes with high yields, but varying sensitivities to environmental conditions have been identified as important check cultivars for testing the next generation of promising genotypes, which is currently

underway. More work is needed in exploring the underlying environmental causes for the observed yields and this will be the focus of a future analysis, which will incorporate an expanded dataset of North American willow yields.

2.6 References

Åhman I (1998) Rust scorings in a plantation of *Salix viminalis* clones during ten consecutive years. *European Journal of Forest Pathology,* 28, 251-258.

Amichev BY, Hangs RD, Belanger N, Volk TA, Vujanovic V, Schoenau JJ, Van Rees KCJ (2015) First-Rotation Yields of 30 Short-Rotation Willow Cultivars in Central Saskatchewan, Canada. *Bioenergy Research,* 8, 292-306.

Annicchiarico P (2002) *Genotype x environment interactions: challenges and opportunities for plant breeding and cultivar recommendations,* Rome, Italy, Food & Agriculture Organization.

Aylott MJ, Casella E, Tubby I, Street NR, Smith P, Taylor G (2008) Yield and spatial supply of bioenergy poplar and willow short-rotation coppice in the UK. *New Phytologist,* 178, 358-370.

Bonosi L, Ghelardini L, Weih M (2013) Towards making willows potential bio-resources in the South: Northern *Salix* hybrids can cope with warm and dry climate when irrigated. *Biomass and Bioenergy,* 51, 136-144.

Buchholz T, Volk TA (2011) Improving the profitability of willow crops-identifying opportunities with a crop budget model. *Bioenergy Research,* 4, 85-95.

Cameron KD, Phillips IS, Kopp RF, Volk TA, Maynard CA, Abrahamson LP, Smart LB (2008) Quantitative genetics of traits indicative of biomass production and heterosis in 34 full-sib F_1 *Salix eriocephala* families. *Bioenergy Research,* 1, 80-90.

Caputo J, Balogh SB, Volk TA, Johnson L, Puettmann M, Lippke B, O'neil E (2014) Incorporating uncertainty into a life cycle assessment (LCA) model of short-rotation willow biomass (*Salix* spp.) crops. *Bioenergy Research,* 7, 48-59.

Cooper M, Hammer GL (1996) Synthesis of strategies for crop improvement. In: *Plant adaptation and crop improvement.* (eds Cooper M, Hammer GL) pp 591-623. Wallingford, UK, CABI.

Eberhart SA, Russell WA (1966) Stability parameters for comparing varieties. *Crop Science,* 6, 36-40.

Environment Canada. (2015). National Climate Data Retrieved 6 October, 2015, from http://climate.weather.gc.ca/

Finlay KW, Wilkinson GN (1963) The analysis of adaptation in a plant-breeding programme. *Crop and Pasture Science,* 14, 742-754.

Gauch HG (1988) Model selection and validation for yield trials with interaction. *Biometrics,* 44, 705-715.

Gauch HG (1992) *Statistical analysis of regional yield trials: AMMI analysis of factorial designs,* Amsterdam, Elsevier.

Gauch HG (2006a) Statistical analysis of yield trials by AMMI and GGE. *Crop Science,* 46, 1488-1500.

Gauch HG (2006b) Winning the accuracy game. *American Scientist,* 94, 133-141.

Gauch HG (2007) MATMODEL Version 3.0: Open source software for AMMI and related analyses. *Crop and Soil Sciences, Cornell University, Ithaca, NY, USA.*

Gauch HG (2012) *Scientific Method in Brief,* New York, Cambridge University Press.

Gauch HG (2013) A simple protocol for AMMI analysis of yield trials. *Crop Science,* 53, 1860-1869.

Gauch HG, Furnas RE (1991) Statistical analysis of yield trials with MATMODEL. *Agronomy Journal,* 83, 916-920.

Gauch HG, Jr., Zobel RW (1990) Imputing missing yield trial data. *Theoretical and Applied Genetics,* 79, 753-761.

Gauch HG, Piepho H-P, Annicchiarico P (2008) Statistical analysis of yield trials by AMMI and GGE: Further considerations. *Crop Science,* 48, 866-889.

Gauch HG, Zobel RW (1997) Identifying mega-environments and targeting genotypes. *Crop Science,* 37, 311-326.

Gollob HF (1968) A statistical model which combines features of factor analytic and analysis of variance techniques. *Psychometrika,* 33, 73-115.

Gouker FE, Smart LB. (2015). Willow cultivar diversity groups fact sheet Retrieved 21 October, 2015, from http://willow.cals.cornell.edu/

Hanley SJ, Karp A (2013) Genetic strategies for dissecting complex traits in biomass willows (*Salix* spp.). *Tree Physiology*.

Hauk S, Knoke T, Wittkopf S (2014) Economic evaluation of short rotation coppice systems for energy from biomass—A review. *Renewable and Sustainable Energy Reviews,* 29, 435-448.

Heller MC, Keoleian GA, Volk TA (2003) Life cycle assessment of a willow bioenergy cropping system. *Biomass and Bioenergy*, 25, 147-165.

Karp A, Hanley SJ, Trybush SO, Macalpine W, Pei M, Shield I (2011) Genetic improvement of willow for bioenergy and biofuels. *Journal of Integrative Plant Biology,* 53, 151-165.

Karp A, Shield I (2008) Bioenergy from plants and the sustainable yield challenge. *New Phytologist,* 179, 15-32.

Keoleian GA, Volk TA (2005) Renewable energy from willow biomass crops: life cycle energy, environmental and economic performance. *Critical Reviews in Plant Sciences*, 24, 385-

406.

Kiernan BD, Volk TA, Tharakan PJ, Nowak CA, Phillipon SP, Abrahamson LP, White EH (2003) Clone-site testing and selections for scale-up plantings. *Final Report prepared for the United States Department of Energy*, SUNY-ESF, Syracuse, NY, 67 pp.

Kopp RF, Smart LB, Maynard CA, Isebrands JG, Tuskan GA, Abrahamson LP (2001) The development of improved willow clones for eastern North America. *Forestry chronicle,* 77, 287-292.

Kuzovkina YA, Quigley MF (2005) Willows beyond wetlands: Uses of *Salix* L. species for environmental projects. *Water Air and Soil Pollution,* 162, 183-204.

Kuzovkina YA, Weih M, Romero MA et al. (2008) *Salix*: Botany and global horticulture. In: *Horticultural Reviews.* (ed Janick J) pp 447-489. John Wiley & Sons, Inc.

Labrecque M, Teodorescu TI (2003) High biomass yield achieved by *Salix* clones in SRIC following two 3-year coppice rotations on abandoned farmland in southern Quebec, Canada. *Biomass and Bioenergy,* 25, 135-146.

Larsen SU, Jørgensen U, Lærke PE (2014) Willow yield is highly dependent on clone and site. *Bioenergy Research,* 7, 1280-1292.

Larsson S (1998) Genetic improvement of willow for short-rotation coppice. *Biomass and Bioenergy,* 15, 23-26.

Lindegaard KN, Barker JHA (1997) Breeding willows for biomass. *Aspects of Applied Biology,* 49, 155-162.

Lindegaard KN, Carter MM, McCracken A et al. (2011) Comparative trials of elite Swedish and UK biomass willow varieties 2001-2010. *Aspects of Applied Biology,* 57-66.

McCracken AR, Dawson WM (2003) Rust disease (Melampsora epitea) of willow (*Salix* spp.)

grown as short rotation coppice (SRC) in inter- and intra-species mixtures. *Annals of Applied Biology,* 143, 381-393.

Mosseler A (1990) Hybrid performance and species crossability relationships in willows (*Salix*). *Canadian Journal of Botany,* 68, 2329-2338.

Mosseler A, Major JE, Labrecque M (2014a) Genetic by environment interactions of two North American *Salix* species assessed for coppice yield and components of growth on three sites of varying quality. *Trees,* 28, 1401-1411.

Mosseler A, Major JE, Labrecque M (2014b) Growth and survival of seven native willow species on highly disturbed coal mine sites in eastern Canada. *Canadian Journal of Forest Research*.

NASA POWER. (2015). Prediction of worldwide energy resource climatology resource for agroclimatology Retrieved 6 October, 2015, from http://power.larc.nasa.gov/

NOAA NCEI. (2015). Global historical climatology network Retrieved 6 October, 2015, from http://www.ncdc.noaa.gov/data-access

Paderewski J, Rodrigues PC (2014) The usefulness of EM-AMMI to study the influence of missing data pattern and application to Polish post-registration winter wheat data. *Australian Journal of Crop Science,* 8, 640-645.

Piepho HP (1995) Robustness of statistical tests for multiplicative terms in the additive main effects and multiplicative interaction model for cultivar trials. *Theoretical and Applied Genetics,* 90, 438-443.

Piepho HP, Möhring J (2005) Best linear unbiased prediction of cultivar effects for subdivided target regions. *Crop Science,* 45, 1151-1159.

Richards BK, Stoof CR, Cary IJ, Woodbury PB (2014) Reporting on marginal lands for

bioenergy feedstock production: a modest proposal. *Bioenergy Research*, 7, 1060-1062.

Rodrigues PC, Pereira DGS, Mexia JT (2011) A comparison between Joint Regression Analysis and the Additive Main and Multiplicative Interaction model: the robustness with increasing amounts of missing data. *Scientia Agricola*, 68, 679-686.

Rönnberg-Wästljung A, Thorsén J (1988) Inter- and intraspecific variation and genotype × site interaction in *Salix Alba* L., *S. Dasyclados* Wimm. and *S. viminalis* L. *Scandinavian Journal of Forest Research*, 3, 449-463.

SAS Institute Inc. (2013) (Version 9.4). Cary, NC, USA: SAS Institute, Inc.

Serapiglia MJ, Cameron KD, Stipanovic AJ, Abrahamson LP, Volk TA, Smart LB (2013) Yield and woody biomass traits of novel shrub willow hybrids at two contrasting sites. *Bioenergy Research*, 6, 533-546.

Serapiglia MJ, Gouker FE, Hart JF, Unda F, Mansfield SD, Stipanovic AJ, Smart LB (2015) Ploidy level affects important biomass traits of novel shrub willow (*Salix*) hybrids. *Bioenergy Research*, 8, 259-269.

Serapiglia MJ, Gouker FE, Smart LB (2014) Early selection of novel triploid hybrids of shrub willow with improved biomass yield relative to diploids. *BMC Plant Biology*, 14, 74.

Simmonds NW (1991) Selection for local adaptation in a plant breeding programme. *Theoretical and Applied Genetics*, 82, 363-367.

Sixto H, Gil P, Ciria P, Camps F, Sánchez M, Cañellas I, Voltas J (2014) Performance of hybrid poplar clones in short rotation coppice in Mediterranean environments: analysis of genotypic stability. *GCB Bioenergy*, 6, 661-671.

Sixto H, Salvia J, Barrio M, Ciria MP, Cañellas I (2011) Genetic variation and genotype-environment interactions in short rotation *Populus* plantations in southern Europe. *New*

Forests, 42, 163-177.

Smart LB, Cameron KD (2008) Genetic improvement of willow (*Salix* spp.) as a dedicated bioenergy crop. In: *Genetic Improvement of Bioenergy Crops.* (ed Vermerris W) pp 377-396. Springer New York.

Smart LB, Cameron KD (2012) Shrub willow. In: *Handbook of Bioenergy Crop Plants.* (eds Kole C, Joshi CP, Shonnard DR) pp 687-708. Boca Raton, FL, Taylor and Francis Group.

Smart LB, Cameron KD, Volk TA, Abrahamson LP (2008) Breeding, selection, and testing of shrub willow as a dedicated energy crop. *NABC Report 19 Agricultural Biofuels: Technology, Sustainability, and Profitability*, National Agricultural Biotechnology Council, Ithaca, NY, 85-92.

Smart LB, Volk TA, Lin J et al. (2005) Genetic improvement of shrub willow (*Salix* spp.) crops for bioenergy and environmental applications in the United States. *Unasylva,* 56, 51-55.

Smith AB, Cullis BR, Thompson R (2005) The analysis of crop cultivar breeding and evaluation trials: an overview of current mixed model approaches. *Journal of Agricultural Science,* 143, 449-462.

Stoof CR, Richards BK, Woodbury PB et al. (2015) Untapped potential: Opportunities and challenges for sustainable bioenergy production from marginal lands in the Northeast USA. *Bioenergy Research,* 8, 482-501.

Stott KG (1991) Nomenclature of the promising biomass coppice willows, *Salix* × *sericans* Tausch ex Kern., *Salix dasyclados* Wimm. and *Salix* ‘Aquatica Gigantea’. *Botanical Journal of Scotland,* 46, 137-143.

U. S. Department of Energy (2011) U.S. billion-ton update: Biomass supply for a bioenergy and bioproducts industry. R.D. Perlack and B.J. Stokes (Leads). Oak Ridge, TN, Oak Ridge

National Laboratory.

Van Eeuwijk F (1995) Linear and bilinear models for the analysis of multi-environment trials: I. An inventory of models. *Euphytica*, 84, 1-7.

Volk TA, Abrahamson LP, Cameron KD et al. (2011) Yields of willow biomass crops across a range of sites in North America. *Aspects of Applied Biology*, 67-74.

Walsh M, De La Torre Ugarte D, Shapouri H, Slinsky S (2003) Bioenergy crop production in the United States: Potential quantities, land use changes, and economic impacts on the agricultural sector. *Environmental and Resource Economics,* 24, 313-333.

Wang DA, Jaiswal D, Lebauer DS, Wertin TM, Bollero GA, Leakey ADB, Long SP (2015) A physiological and biophysical model of coppice willow (*Salix* spp.) production yields for the contiguous USA in current and future climate scenarios. *Plant, Cell & Environment,* 38, 1850-1865.

Wang Z, Macfarlane DW (2012) Evaluating the biomass production of coppiced willow and poplar clones in Michigan, USA, over multiple rotations and different growing conditions. *Biomass and Bioenergy,* 46, 380-388.

Yan W, Hunt LA, Sheng Q, Szlavnics Z (2000) Cultivar evaluation and mega-environment investigation based on the GGE biplot. *Crop Science,* 40, 597-605.

Zalesny RS, Jr., Hall RB, Zalesny JA, Mcmahon BG, Berguson WE, Stanosz GR (2009) Biomass and genotype × environment interactions of *Populus* energy crops in the Midwestern United States. *Bioenergy Research*, 2, 106-122.

Zobel RW, Wright MJ, Gauch HG (1988) Statistical analysis of a yield trial. *Agronomy Journal,* 80, 388-393.

Zsuffa L (1990) Genetic improvement of willows for energy plantations. *Biomass,* 22, 35-47

CHAPTER 3

A mixed model approach for evaluating yield improvements in interspecific hybrids of shrub willow, a dedicated bioenergy crop

Indus. Crops Prod. (2017) 96:57-70

3.1 Abstract

Lignocellulosic bioenergy crops such as shrub willow (*Salix* spp.) are expected to have a significant role in climate mitigation strategies. Yield is perhaps the most important trait for genetic selection and development of biomass feedstocks, but the selection process is complicated by the strong influence that environmental factors has on cultivar performance. Understanding the genotype-by-environment interactions using regional yield trials of shrub willow will inform future breeding efforts and improve the ability to make regionally-specific cultivar recommendations. We analyzed two yield trial datasets containing genotypes from successive rounds of breeding using a series of mixed models. Stability variance parameters were used together with overall yields to identify genotypes with stable yields or specific adaptation. Analysis of the first dataset revealed a group of triploid interspecific hybrid cultivars with exceptional performance and specific adaptation to either poor or good quality environments. The best performing cultivar showed a 14% yield improvement over the mean of the check cultivars. In the second dataset, new selections were assessed along with the top performing cultivars from dataset 1. New triploid hybrids had yields 19 to 27% greater than the check cultivar and exhibited similar patterns of specific adaptation as some of the selections from the first dataset. Partial least squares regression analysis indicated that top-yielding genotypes were favored in environments with higher temperatures and greater growing degree days. This

analysis demonstrates incremental improvements in yield with successive rounds of breeding through the development of interspecific triploid hybrids.

3.2 Introduction

Increasing energy production from renewable, low-carbon sources is an imperative for mitigating the impacts of climate change. Current US national energy policy mandates a rapid expansion of cellulosic biofuel production over the coming years (Environmental Protection Agency 2007). These statutory provisions will likely only be met by increasing the area devoted to growing dedicated bioenergy crops, especially on low productivity agricultural lands. Perennial lignocellulosic feedstocks such as shrub willow (*Salix* spp.) and poplar (*Populus* spp.) managed in short rotation coppice systems are expected to provide a substantial portion of biomass needed in the US to achieve renewable energy goals (U. S. Department of Energy 2011). These crops remain on the landscape for many years, so selecting cultivars that are not well matched to local climatic and edaphic conditions can be a costly mistake to commercial growers.

Shrub willow is a fast-growing, woody perennial C_3 feedstock that is well adapted to humid, temperate regions such as the northeastern and upper midwestern US. It is typically managed on 2-4 year harvest cycles with an expected productive crop life of over 25 years. In addition to supplying large amounts of biomass over short periods of time, shrub willow has low or net negative greenhouse gas emissions (Caputo et al. 2014; Djomo et al. 2015; Wightman et al. 2015), mainly through high rates of C storage in belowground components (Cunniff et al. 2015; Pacaldo et al. 2013; 2014), and has greenhouse gas mitigation potential when converted to bioenergy for displacement of fossil fuel (Djomo et al. 2011). Other features that make shrub willow interesting for breeding are that it is dioecious, is amenable to interspecific hybridization,

has multiple ploidy levels, and can be clonally propagated. Shrub willow re-sprouts vigorously after harvest, has a low nutrient demand and high nutrient use efficiency (Aronsson et al. 2014; Hangs et al. 2014) and a large potential for applications in phytoremediation projects (Kuzovkina and Quigley 2005).

In the US, commercial production of shrub willow for use in biopower production was recently initiated on a small scale in New York State, in part due to a regionally specific federal assistance program (Volk et al. 2016). In Canada, despite decades of research into the production potential of shrub willow, particularly of native species, wide-spread adoption has been hampered by socio-economic issues (Larocque et al. 2013; McKenney et al. 2014). Current economic analyses of short-rotation biomass production including willow suggest that yield is an important factor determining economic viability (Buchholz and Volk 2011; Hauk et al. 2014). Recent breeding efforts in the US have focused largely on improving yields by capturing heterosis from novel interspecific crosses (Smart and Cameron 2012; Smart et al. 2005). Specifically, crosses between diploid and tetraploid species producing triploid progeny have demonstrated substantial yield gains over commercial check cultivars in early stages of evaluation (Serapiglia et al. 2014).

Yield data on short rotation woody crop production in North America is somewhat limited, due largely to its relatively recent reemergence on the continent as a biomass feedstock (Volk et al. 2006). However, recent initiatives aimed at testing newly bred cultivars in regional yield trials have generated valuable datasets for testing genotype-by-environment interactions (GEI) in North America (Serapiglia et al. 2014; Volk et al. 2011). Traditional analysis of variance (ANOVA) approaches to multi-environment datasets have demonstrated significant GEI in shrub willow (Larsen et al. 2014; Mosseler et al. 2014; Serapiglia et al. 2013). However,

these approaches often only test for the presence or absence of statistically significant interactions and thus do little to describe potential structure in the interaction, through which genotype stability or adaptability can be observed. Understanding these nuances is of great importance to breeding programs. Popular approaches to exploring yield stability include regression on environment mean techniques (Eberhart and Russel 1966; Finlay and Wilkinson 1963) or partitioning the total GEI variance among individual contributions from each genotype (Shukla 1972). While the regression approach is intuitive, it is often criticized for capturing too little of the GEI variance (Crossa 1990). Gollob (1968) suggested combining ANOVA with factor analytic decomposition of the interaction matrix in order to reduce noise and find structure among the interacting factors. Gauch (1988) popularized this approach for crop yield trial data with the so-called Additive Main Effects and Multiplicative Interactions (AMMI) model. The AMMI model is a powerful technique for interaction noise reduction, resulting in the assignment of interaction scores to each genotypes which are accurate measures of yield stability or adaptability. In a recent analysis of shrub willow yield trials across North America, Fabio et al. (2017) used the AMMI model to confirm the superior performance of a group of triploid hybrids, which were identified as having greater yields and exhibiting differential patterns of broad and narrow adaptability. That evaluation was restricted, however, to 16 genotypes present in 10 environments. This restriction was imposed in order to satisfy the need of a balanced dataset when using fixed-effects AMMI.

More recent efforts have demonstrated the utility of mixed effects models for the analysis of GEI datasets because they offer a flexible framework for exploring the GEI, specifically through the application of different variance-covariance structures, which impose certain assumptions about the complexity of the random factor variances (Hu and Spilke 2011; Sixto et

al. 2014). The mixed model approach can handle unbalanced datasets, which are common in yield trial evaluations and can also incorporate heterogeneity of variances at multiple levels (Piepho et al. 2003; Smith et al. 2005; Raman et al. 2011).

In this study we present first rotation data from two sets of yield trials representing early and more recent stages of genetic improvement in shrub willow. Because not all genotypes evaluated were present in every test environment, the mixed model approach allowed us to incorporate a much greater number of observations available in the datasets than would otherwise allow in a fixed-effects framework. The objectives were 1) to test a series of mixed models on an existing dataset of shrub willow yields (Dataset 1) to identify stable and regionally adapted cultivars, 2) to analyze a new dataset (Dataset 2) of improved genotypes to identify yield improvements after successive breeding and selection and 3) to use environmental covariates to explain patterns in genotypic sensitivities to growing conditions in both datasets.

3.3 Materials and methods

3.3.1 Yield Trial Dataset 1

Breeding material consisted of native and naturalized accessions of *Salix eriocephala* and *S. purpurea*, collected from the northeastern US and eastern Canada, in combination with germplasm secured from the University of Toronto, consisting mainly of improved *S. eriocephala* and natural collections of *S. miyabeana*, native to East Asia, as well as *S. viminalis*, a native of Europe. Controlled intraspecific and interspecific crosses were performed at the State University of New York College of Environmental Science and Forestry, mostly in 1998 and 1999. Progeny from each successful cross were planted in nursery beds and individuals were selected based on growth traits for evaluation in a replicated selection field trial in Tully, New

York in 2002 (Smart et al. 2008). Top-performing genotypes across multiple pedigrees were advanced to multi-location yield trials (Table 3.1).

Table 3.1 Characteristics of the 21 shrub willow cultivars present in Dataset 1.

Epithet[a]	Species/Pedigree	Mother	Father	Diversity Group[b]	Sex	Ploidy[c]	Program/ origin	No. of trials
SV1	*S.* × *dasyclados*			1	F	2X	Unknown[d]	15
S25	*S. eriocephala*	*S. erio* 16	*S. erio* 276	4	F	2X	Canada	13
Allegany	*S. koriyanagi* × *S. purpurea*	SH3	95058	6b	F	2X	USA	14
Onondaga	*S. koriyanagi* × *S. purpurea*	SH3	94002	6b	M	2X	USA	14
94001	*S. purpurea*			6a	M	2X	Natural accession	11
Fish Creek	*S. purpurea*	94006	94001	6a	M	2X	USA	15
Wolcott	*S. purpurea*	94006	94001	6a	F	2X	USA	10
Millbrook	*S. purpurea* × *S. miyabeana*	95026	SX64	9	F	3X	USA	15
Oneida	*S. purpurea* × *S. miyabeana*	94006	SX67	9	M	3X	USA	15
Oneonta	*S. purpurea* × *S. miyabeana*	94006	SX64	9	M	3X	USA	12
Fabius	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X	USA	13
Otisco	*S. viminalis* × *S. miyabeana*	SV2	SX64	8	F	3X	USA	15
Owasco	*S. viminalis* × *S. miyabeana*	SV7	SX64	8	F	3X	USA	14
Truxton	*S. viminalis* × *S. miyabeana*	SV7	SX64	8	M	3X	USA	12
Tully Champion	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X	USA	14
Canastota	*S. miyabeana*	SX61	SX64	5	M	4X	USA	14
Cicero	*S. miyabeana*	SX61	SX64	5	F	4X	USA	10
Marcy	*S. miyabeana*	SX61	SX64	5	F	4X	USA	10
Sherburne	*S. miyabeana*	SX61	SX67	5	F	4X	USA	14
SX61	*S. miyabeana*			5	F	4X	Natural accession	15
SX64	*S. miyabeana*			5	M	4X	Natural accession	15

[a]The three check cultivars are underlined

[b]Diversity group codes are a shorthand reference to cultivar species/pedigrees

[c]Ploidy level estimated by flow cytometry (Serapiglia et al. 2015)

[d]Collected in Ontario Canada, but possibly an escaped horticultural hybrid of *S. viminalis* × *S. caprea* × *S. cinerea* (Stott 1991)

These yield trials were established between 2005 and 2011 and were hosted by eight institutions across six US states and two Canadian Provinces (Table 3.2). For detailed descriptions of trial establishment and design see Serapiglia et al. (2013) and Fabio et al. (2017). Planting material for each trial originated as 25-cm dormant, hardwood cuttings sourced from nursery beds at the SUNY-ESF Genetics Field Station in Tully, NY. After conventional site preparation, cuttings were planted by hand, usually in May or June, in a double-row configuration at a planting density of approximately 14,400 plants ha^{-1}. The distance between plants within a row was 0.61 m, between adjacent rows 0.76 m, and between double rows 1.52 m (alleys). Experimental units consisted of plots planted with a single cultivar, three double rows wide and 13 plants long. The outer two double rows were considered guard rows and only the middle double row was used for measurements in order to avoid edge effects. Each trial was planted with between 18 and 30 genotypes in a randomized complete block design, with four replicates of each genotype. Three check cultivars, 'SV1', 'SX61' and 'SX64' were used throughout testing and at least two checks were present in each trial.

After the first year of growth all aboveground biomass was cutback during dormancy close to ground level to promote a multi-stem coppice regrowth response in the following spring. At that time each trial received a single application of 112 kg N ha^{-1} applied as ammonium sulfate, except for the Saskatchewan trial, which received no fertilizer during the study period. Trials were allowed to grow for three years after cutback, at which time 18-22 plants from the middle double row of each plot were harvested. Harvests were performed either manually by cutting whole stems at ground level with a brush saw and weighed with spring scales, or mechanically in a single-pass cut and chip operation using a forage harvester fitted with a specialized cutting header, where the chips were blown into a weigh bin with load cells.

Table 3.2 Yield trial locations and environmental covariates for Dataset 1.

Location	Code	Host institution	Year planted	LAT	LONG	Elev	Prc	GDD	Tmax	Tmin
						(m)	(mm)	(°C)	(°C)	(°C)
Albion, MI	Albi	Michigan St Univ	2011	42.19	-84.73	312	674	1313	21	2
Belleville, NY	Bell	SUNY ESF	2005	41.98	-78.56	145	602	1274	21	2
Boisbriand, QU	Bois	Univ Montreal	2007	45.63	-73.89	30	651	1162	20	1.2
Brimley, MI	Brim	Michigan St Univ	2009	46.40	-84.47	200	564	1021	19	1.7
Constableville, NY	Cons	SUNY ESF	2006	43.56	-75.53	513	842	812	19	-1.7
Escanaba, MI	Esca	Michigan St Univ	2008	45.77	-87.20	222	537	1016	19	-0.1
Fredonia, NY	Fred	Cornell Univ	2008	42.44	-79.29	255	662	1477	21	4.9
Middlebury, VT	Midd	SUNY ESF	2007	44.01	-73.20	114	766	1419	23	2.0
Potsdam, NY	Pots	SUNY Potsdam	2009	44.67	-74.97	134	714	1318	21	2
Saskatoon, SK	Sask	Univ Saskatchewan	2007	52.13	-106.61	510	345	767	18	-4.1
Savoy, IL	Savo	Univ Illinois	2010	40.07	-88.20	224	618	2097	25	6
Skandia, MI	Skan	Michigan St Univ	2009	46.36	-87.24	287	581	870	18	0.1
Storrs, CT	Stor	Univ Connecticut	2009	41.80	-72.23	198	848	1487	21	5.4
Tully, NY	Tull	SUNY ESF	2005	42.79	-76.12	379	816	1155	20	2
Waseca, MN	Wase	Univ Minn	2006	44.06	-93.54	349	653	1459	22	1.7

LAT, Latitude (decimal degrees); LONG, longitude (decimal degrees); Elev, elevation; Prc, growing season (April-Oct) precipitation Y; GDD, annual growing degree days (base 10 ˚C); Tmax, growing season mean maximum temperature (˚C); Tmin, annual mean minimum temperature (˚C).

Table 3.2 Continued

Location	Code	Rad (MJ m^{-1} day^{-1})	SOM (%)	pH	soilP (mg kg^{-1})	soilK (mg kg^{-1})
Albion, MI	Albi	3790	2.0	6.37	5.6	128.3
Belleville, NY	Bell	3957	3.3	6.49	7.8	109.4
Boisbriand, QU	Bois	3576	4.0	6.09	1.0	48.2
Brimley, MI	Brim	3882	4.0	5.25	1.0	69.6
Constableville, NY	Cons	3572	8.2	5.66	1.0	71.8
Escanaba, MI	Esca	4008	2.8	6.10	1.0	30.3
Fredonia, NY	Fred	3895	3.6	4.80	8.3	122.5
Middlebury, VT	Midd	3548	6.8	6.70	27.3	150.4
Potsdam, NY	Pots	3470	6.8	5.65	1.5	48.8
Saskatoon, SK	Sask	3535	4.5	7.07	10.6	326.5
Savoy, IL	Savo	3977	2.4	6.27	1.0	84.7
Skandia, MI	Skan	3865	3.6	6.47	1.0	41.5
Storrs, CT	Stor	3785	3.4	6.12	1.2	72.8
Tully, NY	Tull	3674	3.2	4.99	2.4	77.2
Waseca, MN	Wase	3802	5.7	5.40	42.9	216.9

Rad, growing season solar radiation; SOM, soil organic matter; pH, soil pH; SoilP, soil phosphorus; SoilK, soil potassium. Climatic variables are four-year means across the first harvest rotation.

A subsample of whole or chipped stems from each plot were weighed fresh, oven dried at 65 ˚C to a constant mass, then weighed again in order to calculate moisture content. Moisture content was used to express all plot weights as oven-dried Mg of biomass per ha per year. Prior to harvest, survival was scored on all harvested plants within a plot and any plot with <65% survival excluded from analysis. This left 1,063 yield observations out of a possible 1,260. In total 21 genotypes were tested across 15 locations, with 35 (11%) missing cells in the two-way genotype-by-environment table of means.

3.3.2 Yield Trial Dataset 2

A second round of cultivar testing was initiated in 2012 and 2013, based on top-performing genotypes from diverse pedigrees in a genetic selection trial established in 2008 at Cornell University in Geneva, NY (Serapiglia et al. 2014). Material tested was a combination of commercially available cultivars, many of which were present in Dataset 1, as well as improved genotypes whose pedigrees include commercial cultivars and accessions from the US and Europe (Table 3.3). Trials were randomized complete block designs established in four locations: Escanaba, MI, Fredonia, NY, Geneva, NY and Rock Springs, PA (Table 3.4). The Fredonia, NY location consisted of two side-by-side trials, one receiving 1,800 kg ha^{-1} of lime and 84 kg N, P and K ha^{-1} in both the establishment year and first year post-coppice, and Geneva, NY received 160 kg N and 87 kg P ha^{-1} in the first year post-coppice, while Escanaba, MI and Rock Springs, PA received no soil amendments during the study period. For the purpose of GEI modeling in this study, the two trials in Fredonia, NY were considered as separate environments. Planting material was sourced from nursery beds at the New York State Agricultural Experiment Station (Geneva, NY) and from Double A Willow nursery (Fredonia, NY).

Table 3.3 Characteristics of the 25 shrub willow genotypes present in Dataset 2.

Clone ID/ Epithet	Species/Pedigree	Mother	Father	Diversity Group[a]	Sex	Ploidy[b]	Program/ origin[c]	No. of trials
94006	*S. purpurea*			6a	F	2X	Nat acc	5
05X-293-047	*S. purpurea*	05-01-002	Fish Creek	6a	M	2X	USA	3
Fish Creek	*S. purpurea*	94006	94001	6a	M	2X	USA	5
01X-265-019	*S. koriyanagi* × *S. purpurea*	SH3	94001	6b	M	2X	USA	5
Dimitrios	*(S. schwerinii* × *S. viminalis)* × *S. aeygyptiaca*	Tora		14	M	3X	Sweden	5
02X-326-010	*S. miyabeana* × *(S. schwerinii* ×. *S. viminalis)*	9970-021	Olof	10	M	3X	USA	5
02X-326-015	*S. miyabeana* × *(S. schwerinii* ×. *S. viminalis)*	9970-021	Olof	10	M	3X	USA	5
Victor	*S. purpurea* × *S. miyabeana*	00-01-088	SX67	9	F	3X	USA	5
05X-291-050	*S. purpurea* × *S. miyabeana*	00-01-088	SX67	9	M	3X	USA	5
Sterling	*S. purpurea* × *S. miyabeana*	05-01-002	SX64	9	M	3X	USA	3
Hopewell	*S. purpurea* × *S. miyabeana*	05-01-002	SX64	9	F	3X	USA	3
Millbrook	*S. purpurea* × *S. miyabeana*	95026	SX64	9	F	3X	USA	5
Saratoga	*S. purpurea* × *S. miyabeana*	95026	SX64	9	F	3X	USA	5
Tonawanda	*(S. koriyanagi* × *S. purpurea)* × *S. miyabeana*	99239-015	SX67	9b	F	3X	USA	5
Geneva Giant	*(S. koriyanagi* × *S. purpurea)* × *S. miyabeana*	99239-015	SX67	9b	F	3X	USA	5
Sheridan	*S. viminalis* × *(S. viminalis* × *S. miyabeana)*	SV2	99207-019	8	F	3X	USA	5
Seneca Chief	*S. viminalis* × *S. miyabeana*	SV7	9970-037	8	F	3X	USA	5
Fabius	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X	USA	5
LA970253	*S. viminalis* × *S. miyabeana*			8	F	3X	UK	4
Otisco	*S. viminalis* × *S. miyabeana*	SV2	SX64	8	F	3X	USA	5
Preble	*S. viminalis* × *S. miyabeana*	SV2	9970-037	8	F	3X	USA	5
Tully Champion	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X	USA	3
Canastota	*S. miyabeana*	SX61	SX64	5	M	4X	USA	3
SX61	*S. miyabeana*			5	F	4X	Nat acc	5
India	*S. dasyclados*			15	M	6X	Canada	5

[a]Diversity group codes are a direct reference to cultivar species/pedigree

[b] Ploidy level estimated by flow cytometry (Serapiglia et al. 2015)

[c]Nat acc, natural accession

Table 3.4 Yield trial locations and environmental covariates for Dataset 2.

		Escanaba, MI	Fredonia, NY Amended	Fredonia, NY Control	Geneva, NY	Rock Springs, PA
LAT		45.77	42.44	42.44	42.88	40.70
LONG		-87.20	-79.29	-79.29	-77.00	-77.96
Elev	(m)	219.1	255.9	255.9	167.0	403.7
Prc	(mm)	515.2	658.9	658.9	621.2	744.3
GDD	(°C)	981.7	1431.3	1431.3	1466.6	1651.5
Tmax	(°C)	19.3	20.9	20.9	21.4	22.0
Tmin	(°C)	-0.2	4.1	4.1	4.1	5.6
Rad	(MJ m^{-1} day^{-1})	3723.8	3624.1	3624.1	3513.7	3484.4
SOM	(%)	2.5	4.1	3.8	2.3	4.7
pH		7.0	6.2	6.0	6.5	5.6
soilP	(mg kg^{-1})	11.8	1.1	1.0	1.0	1.0
soilK	(mg kg^{-1})	71.1	104.6	83.9	44.7	90.6
soilCa	(mg kg^{-1})	1344.4	1526.9	1193.5	1414.6	1163.4
SoilAl	(mg kg^{-1})	9.8	55.3	48.9	21.2	44.4

LAT, Latitude (decimal degrees); LONG, longitude (decimal degrees); Elev, elevation; Prc, growing season (April-Oct) precipitation; GDD, annual growing degree days (base 10 °C); Tmax, growing season mean maximum temperature; Tmin, annual mean minimum temperature; Rad, growing season solar radiation; SOM, soil organic matter ; pH, soil pH; SoilP, soil Phosphorus; SoilK, soil potassium. Climatic variables are four-year means across the first harvest rotation.

Trials were established in a similar manner as those in Dataset 1 with a slightly modified double-row planting design, where plots contained 48 plants, 24 plants in the middle double row and outer double rows were shared with adjacent plots. One exception was at Geneva, NY, which was planted using two single rows per plot, 12 plants in length, and spaced 1.83 meters apart. Within row distances were adjusted to maintain the same plant density as the double row trials. Geneva and Fredonia, NY trials were harvested two years post-coppice, while Escanaba, MI and Rock Springs, PA were harvested three years post-coppice. At each harvest 16-20 plants were cut and chipped in a single pass and the chips were weighed, and a subsample was collected to determine moisture content. Dataset 2 consisted of 25 genotypes across 5 locations, and the check cultivar used was 'SX61'. The same 65% plant survival per plot criterion as in Dataset 1 was applied so that Dataset 2 consisted of 448 out of a possible 500 observations. There were 114 out of 125 possible genotype-by-environment combinations, or 8% missing cells in the two-way table of means.

3.3.3 Environmental Characterization

Daily temperature and precipitation records were obtained from weather stations based on completeness of records and proximity to trial locations. Data from the National Oceanic and Atmospheric Agency, National Centers for Environmental Information (NOAA NCEI 2015) was used for all US trials and Canadian National Climate Data (Environment Canada 2015) for the two CA trials in Dataset 1. Daily solar radiation (MJ m^{-2} d^{-1}) was obtained from the National Aeronautics and Space Administration's Prediction of Worldwide Energy Resource (NASA POWER 2015), given at a 1^{o} by 1^{o} grid scale. Soil samples at each location were generally collected at the time of planting, or occasionally soon after harvest. Some regional differences in soil sampling methods existed for Dataset 1. All soil samples obtained from trials in Dataset 2

were collected at the time of establishment and extractable nutrients were analyzed by Dairy One (Ithaca, NY) using the Morgan extraction method (Sims and Wolf 1995). Soil pH was assessed in a 1:1 soil/water ratio by weight and % organic matter was determined by loss on ignition.

3.3.4 Statistical Analysis

Datasets 1 and 2 were analyzed separately using single-stage mixed model GEI analyses with PROC MIXED in SAS (SAS Institute Inc. 2013). Genotypes had undergone multiple rounds of selection and so were considered fixed effects. Environments were considered random effects, as was the effect of block, nested within environment. Because environment is considered random, interaction terms become random effects as well. The general form of the linear model fit to our yield datasets is shown in Eq. (1):

$$Y_{ijk} = \mu + G_i + E_j + B_{kj} + (GE)_{ij} + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} is the yield observation for the *i*th genotype in the *k*th block for the *j*th environment, μ is the overall mean, G_i is the effect of the *i*th genotype, E_j is the effect of the *j*th environment, B_{kj} is the effect of the *k*th block (replicate) in the *j*th environment, $(GE)_{ij}$ is the interaction effect of the *i*th genotype in the *j*th environment, and ε_{ijk} is the residual error term for the *i*th genotype in the *k*th block of the *j*th environment.

We tested a series of mixed models which implement different variance-covariance structures in order to model random effects, with particular attention given to the genotype-within-environment effects. A realistic situation for multi-trial data would be that each genotype in each environment has a unique variance estimate, and the modeled variance-covariance structure would be considered completely unstructured. However, with large datasets this type of structure is difficult to implement, since such a large number of variance parameters need to be

estimated. Alternatively, simplified variance-covariance structures can be implemented that make certain assumptions about the degree of complexity associated with the random GEI effects and thus result in different numbers of parameters to be estimated. The resulting variance parameter estimates from these simplified structures are closely related to classical stability parameters, but in a mixed model formulation (Piepho 1999).

Each model is described below in terms their expectations (fixed effects) and variances (random effects). Each model contained two sources of error variance, block within environment (σ^2_{blk}) and residual error variance (σ^2_{ε}), and for simplicity we have excluded these parameters from the descriptions of the models. All variance components were estimated using the REML procedure as implemented by default in SAS PROC MIXED.

(a) *Simple additive model*. Following Denis et al. (1997) this model assumes no interaction effects between the main effects of genotype and environment, and thus the $(GE)_{ij}$ term in Eq. (1) is dropped from the model. This model has a simple variance-covariance structure with the expectation and variance defined as

$$E(Y_{ij}) = G_i;\ Var(Y_{ij}) = \sigma^2_E$$

Where Y_{ij} is the yield of the *i*th genotype in the *j*th environment, and is defined by the fixed effect of genotype, G_i. The variance is defined by one parameter, σ^2_E, which represents the random effect of environment. With this model it is assumed that there is no covariance between genotypes. Since most crop systems exhibit the presence of some GEI effects, this model represents an unlikely scenario, but it can be used as a baseline for interpreting the importance of GEI effects in subsequent models with higher degrees of complexity in modeling the interaction.

(b) *Compound symmetry model*. This model is synonymous with a standard two-way ANOVA with interaction, assuming constant variances for random effects. In this model there are two main diagonal elements of variance-covariance matrix that all genotypes share, the environment main effect, σ^2_E, and the interaction effect, σ^2_{GE}, as well as a common covariance in the off-diagonals, σ^2_{GE} (Hu 2014). This model is defined as

$$E(Y_{ij}) = G_i;\ Var(Y_{ij}) = \sigma^2_E + \sigma^2_{GE}$$

This model assumes that there is no difference in genotypic stability variance parameters, and has just two variance components to estimate.

(c) *Heterogeneous compound symmetry model*. This is a form of the compound symmetry model that attempts to model GEI by allowing for unique genotype-within-environment variances, σ^2_{GEi}. The covariance between two genotypes is defined by $\sigma_{GE1}\sigma_{GE2}\rho$, which is the product of the square roots of two specific variances and a common correlation coefficient, ρ, whose value is less than one (Raman et al. 2011). sd The number of parameters to be estimated becomes $I + 1$, representing the I number genotypes tested plus the common correlation coefficient.

(d) *Shukla's stability variance model*. This represents a mixed model version of the classic stability analysis proposed by Shukla (1972). It is a generalized extension of

$$E(Y_{ij}) = G_i;\ Var(Y_{ij}) = \sigma^2_E + \sigma^2_{GEi}$$

Here each genotype is modeled as having a specific interaction variance, σ^2_{GEi}. The interpretation of these genotype-specific variances is that smaller variances equate to more stable yields for a given genotype across test environments. A banded main diagonal variance-covariance structure

is implemented in this model by specifying the 'type = UN' option in the random statement (Hu and Spilke 2011); however, in our case, there were problems with convergence, so the effect of genotype was added to the GROUP = statement in SAS PROC MIXED and the PARMS statement was used to initiate starting values at one for all variance components (Piepho 1999). This model has $I + 1$ parameters to be estimated.

(e) *Finlay-Wilkinson regression model*. This is another mixed model version of a classic stability analysis (Finlay and Wilkinson 1963), where the genotype-specific interaction parameter is modeled as a regression on the environmental mean in the original fixed effects case. The mixed model version of this analysis can be implemented using a factor analytic (FA) variance-covariance structure (Piepho 1997; 1999). The interaction in Eq. (1) is modeled as a series of multiplicative terms:

$$\sigma^2_{GEi} = \sum_{m=1}^{M} \lambda_{im}\omega_{jm} + \delta_{ij}$$

where λ_i represents genotype factor loadings on a latent environmental variable, ω_j, and the residual term δ_{ij} contains the unexplained interaction. The number of possible multiplicative terms is equal to one minus the number of genotypes or environments, whichever is least. In practice, since the first few factors explain the most variation, a more parsimonious model with one or two factors is considered adequate for explaining the interaction, and the remaining variability is pooled in the residual. In the mixed model Finlay-Wilkinson (F-W) analysis using the FA1(1) variance-covariance structure, no environment main effect is fitted. The multiplicative term is overparameterized, so the identifiability constraint that $\sigma^2_\omega = 1$ is implemented (Piepho 1999). Interpretation of λ_i's in terms of genotype stability or adaptability to environmental conditions is that a genotype with an average λ_i would be considered stable. A

genotype with a relatively large λ_i would be well adapted to high-yielding environments, where as a genotype with relatively a small λ_i could be considered well adapted to low yielding environments (Piepho 1998). This model has $I + 1$ number of variance parameters, including a common residual variance, σ^2_δ, associated with the FA1(1) structure.

(f) *Eberhart-Russell regression model*. This is a mixed model type of the Eberhart-Russell (E-R) fixed-effects model (Eberhart and Russell 1966), and is a nested case of the Finlay-Wilkinson model. The variance-covariance structure implemented for the E-R model is the FA(1) type, which allows for heterogeneity in interaction variance (Sixto et al. 2014). Therefore a genotype-specific deviation variance, $\sigma^2_{\delta i}$, is assigned to each genotype along with a specific loading score, λ_i. The values of λ_i are interpreted in the same way as for the F-W model, but the deviation from regression variance component is an added measure of stability much like that of Shukla's model. The number of GEI parameters that need to be estimated becomes 2*I*.

(g) *Additive Main Effects and Multiplicative Interactions (AMMI) model*. The AMMI model was popularized by Gauch (1988), and is in a similar class of models as F-W and E-R in that the interaction is modeled by a set of multiplicative factors. Here the interaction is modeled with the same factor analytic structure as the F-W model, FA1(*n*), where n is the number of factors included in the model . Similar to the fixed-effects version we refer here to AMMI models according to the number of multiplicative parameters included, such that AMMI-1 includes one multiplicative factor (FA1(1)), and AMMI-2 includes two factors (FA1(2)). In addition to inclusion of $n > 1$ factors, AMMI differs from F-W and E-R models in that the random main effect of environment is included in in the model. As with the F-W and E-R models, the $\lambda_{i,n}$'s for each genotype can be interpreted as different sensitivities to environmental conditions. The number of variance parameters estimated are $I \times n + 2$, including the main effect

of environment and a residual term from the FA1 structure. For our datasets we had convergence issues implementing the FA1(*n*) structure, and so we used the "scoring = *n*" option in the PROC MIXED statement which switches from the default Newton-Raphson to the Fisher scoring method for maximum likelihood estimation (Piepho 1999).

Finally, for each of the models outlined above, we also considered separate cases of heterogeneous residual error variance. The common, pooled homogeneous error estimate is therefore replaced by a separate estimate for each environment, σ^2_{RESj}. Heterogeneous error variances among environments is probably a more realistic scenario given the variable nature of multi-environment trial data (Raman et al. 2011) and given our unbalanced dataset.

Fit statistics including the residual log likelihood (-2 Res LL) and the Akaike information criterion (AIC) were used to select models with the best fit within datasets. Both fit statistics are of the smaller is better type, but the AIC imposes a penalty for number of parameters, given as -2 ResLL + 2*p*, where *p* is the number of variance parameters estimated in the model. Log likelihood ratio tests were also calculated to assess the model fit improvement for homogeneous vs. heterogeneous residual error variances.

3.3.5 Interaction Biplots

Biplots display two different types of data in a combined graphic and have become very useful for interpreting GEI visually. In the case of AMMI models, scores from the first multiplicative factor can be combined with estimates of the main effects to construct so-called AMMI-1 biplots, or scores from the first two multiplicative factors can be combined for AMMI-2 biplots. In order to produce estimates of genotype and environment interaction scores for our unbalanced datasets, following Kumar et al. (2012), we first generated the Best Linear Unbiased Predictors

(BLUPs) for the AMMI models with the FA1(*n*) variance-covariance structures. The BLUPs were then transferred into a now complete table of genotype-by-environment estimates, which was first environment centered, and then subjected to singular-value decomposition using PROC ILM in SAS.

3.3.6 Partial Least Squares Regression

To evaluate GEI in the context of environmental covariates collected for each trial location, we performed partial least squares (PLS) regression using PROC PLS in SAS. In similar fashion to the AMMI biplot construction, since the datasets were unbalanced, we first generated BLUPs to form a complete two-way table of GEI estimates. The compound symmetry variance-covariance model was used to generate BLUPs for both datasets separately. These estimates were subjected to a main-effects ANOVA to produce a table of residuals which contain the GEI effect. Since the PLS procedure is sensitive to scale, estimates were standardized to have a mean of zero and variance of one prior to analysis following Crossa et al. (2015). Biplots were constructed using the first and second PLS factors to illustrate relationships between environment scores and genotype and covariate loadings.

3.4 Results

3.4.1 Yield Trial Dataset 1

The mixed model analyses of Dataset 1 with 21 genotypes and 15 environments indicated that models with larger numbers of variance parameters tended to provide the best fit (Table 3.5). For homogeneous residual error variance scenarios, the AMMI-2 and E-R models provided the best fit to the data based on residual log likelihood. Based on AIC, which imposes a penalty for the number of parameters estimated, AMMI-1 and AMMI-2 models had the best fit. This is

interesting since the AMMI-2 model had the most parameters to be estimated, with one additional parameter compared to the E-R model.

Table 3.5 Fit statistics for mixed models tested in Dataset 1.

	Homogeneous Error Variance			Heterogeneous Error Variance			Res LL[a] Ratio Tests		
Model	***p*[b]**	**-2 Res LL**	**AIC[c]**	***p***	**-2 Res LL**	**AIC**	**X^2**	**DF**	***P*-value**
Simple additive	3	4372.9	4378.9	17	4120.2	4154.2	252.7	14	<0.0001
Compound symmetry	4	4292.7	4300.7	18	4052.4	4088.4	240.3	14	<0.0001
Heterogeneous CS	24	4256.9	4304.9	38	3999.6	4075.6	257.3	14	<0.0001
Shukla's	24	4259.9	4301.9	38	4018.6	4090.6	241.3	14	<0.0001
Finlay-Wilkinson	24	4255.7	4303.7	38	3995.8	4071.8	259.9	14	<0.0001
Eberhart-Russell	44	**4212.9**	4290.9	58	**3953.8**	**4051.8**	259.1	14	<0.0001
AMMI-1	25	4238.9	**4288.9**	39	3988.3	4066.3	250.6	14	<0.0001
AMMI-2	45	**4196.8**	**4286.8**	59	**3946.5**	**4064.5**	250.3	14	<0.0001

[a]Res LL, Residual log likelihood ratio tests, where the Chi-squared statistic is the difference in the -2 log likelihood, and degrees of freedom (DF) is the difference in the number of parameters, between homogeneous and heterogeneous cases.

[b]Number of parameters estimated in the variance-covariance for each model including the effect of block (nested within environment).

[c]AIC, Akaike information criterion. Bold and underlined values of -2 Res LL and AIC indicate best model fit.

Also, for the E-R model, five genotype-specific residual variances were estimated as zero, which as a result do not contribute to the penalty for the number of model parameters in the calculation of the AIC value.

In every case when residual error variances were considered to be heterogeneous for environments, model fit improved significantly ($P < 0.0001$) over the homogeneous models based on the residual log likelihood ratio Chi-squared tests (Table 3.5). Among these scenarios, the E-R and AMMI-2 models provided the best fit to Dataset 1. Although the AIC for the E-R model was lower than for AMMI-2, nine genotype-specific residual variances ($\boldsymbol{\sigma}^2_i$) were estimated at zero, and consequently were not included in the AIC calculation.

The E-R and AMMI-2 models with heterogeneous residual error variances were selected to represent genotype least squared mean yields and stability parameters (Table 3.6). Genotype yield rankings between the two models were very similar (Spearman's r = 0.98). According to the E-R model, 'Fabius' had the greatest overall yield and an above average regression coefficient, suggesting that it was better adapted to high-yielding environments. Cultivars with above average yields and below average regression coefficients were 'Oneonta' and 'Tully Champion', and could be thought of as better adapted to low-yielding environments. 'SX64' and 'Marcy' had above average yields and regression coefficients close to the mean, suggesting that they had relatively stable yields. Since nine out of the 21 genotype-specific residual variances were estimated to be zero according to the E-R model, it could be assumed that the FA(1) structure fit the data well for these genotypes, and they could be considered to be stable. While most of the genotypes with zero residual variances in the E-R model were below average in yield, 'Marcy', 'Fabius' and 'Oneonta' had combined high mean yields and zero or small genotype-specific residual variances. 'Tully Champion' had the second greatest specific residual

variance, while ‘SV1’ had the greatest, suggesting a poor fit to the factor analytic variance-covariance structure.

For the AMMI-2 model ‘Fabius’, ‘Oneonta’ and ‘SX64’ were again among the top yielding genotypes (Table 3.6). The λ_i’s for the first factor of the AMMI-2 model were highly correlated with the E-R model regression coefficients (Pearson’s r = 0.93, $P < 0.0001$). A biplot based on the BLUPs from the AMMI-2 model allows visualizing the genotype and environment interaction (Figure 3.1). Environments on the left side of the figure were among the highest yielding, and some of the top-performing genotypes group with these environments. The environments in the lower-right of the figure were the lowest yielding environments, and the lowest yielding genotype, ‘S25’ falls in the same region, as does ‘Tully Champion’, however its mean yield was relatively greater, suggesting again that it is well adapted to low-yielding environments. ‘Oneida’ and ‘Millbrook’ lie close to the origin, suggesting more stable yields across environments. The genotypes also group according to pedigree and ploidy level, with *S. miyabeana* (tetraploid) and *S. viminalis* × *S. miyabeana* (triploid) genotypes clustered on the left-hand side of the figure, and *S. purpurea* (diploid) and *S. koriyanagi* × *S. purpurea* (diploid) genotypes clustered mainly on the right. Of the three check cultivars, ‘SV1’, ‘SX61’ and ‘SX64’, the latter two are *S. miyabeana* tetraploids, and were shown to have above average yields and somewhat stable interaction variance parameters. However, ‘SV1’ (diploid) had a below average overall yield and large interaction variance parameters, suggesting it is a poor candidate for a check in future testing.

Table 3.6 Variance parameters for best fitting models under heterogeneous residual error variance according to AIC for Dataset 1.

	Eberhart-Russell						AMMI-2					
Genotype	**LS Mean**	**SE**	**λ_i**	**SE**	**σ^2_i**	**SE**	**LS Mean**	**SE**	**$\lambda_{i,1}$**	**SE**	**$\lambda_{i,2}$**	**SE**
Fabius	8.29	1.03	3.96	0.79	0.09	0.15	8.08	0.95	3.08	1.01	0.86	0.70
Oneonta	7.51	0.89	3.40	0.69	0.10	0.21	7.52	0.89	2.71	0.91	1.00	0.45
SX64	7.50	0.86	3.24	0.67	0.28	0.43	7.49	0.85	2.49	0.89	1.05	0.42
Marcy	7.47	1.03	3.97	0.79	0	.	7.32	0.98	3.12	1.01	1.14	0.68
Tully Champion	7.44	0.77	2.69	0.63	1.12	0.74	7.35	0.68	1.67	0.83	0.72	0.44
Canastota	7.42	0.92	3.52	0.70	0	.	7.43	0.90	2.74	0.96	1.19	0.49
SX61	7.36	0.86	3.27	0.67	0.22	0.23	7.44	0.85	2.57	0.88	0.81	0.41
Oneida	7.26	0.82	3.10	0.63	0.13	0.23	7.28	0.83	2.56	0.81	0.41	0.40
Otisco	7.25	0.75	2.78	0.59	0.37	0.39	7.24	0.74	2.02	0.83	0.81	0.38
Fish Creek	7.18	0.93	3.58	0.71	0	.	7.20	0.91	2.98	0.89	-0.04	0.46
Millbrook	7.17	0.83	3.17	0.63	0	.	7.21	0.83	2.59	0.83	0.36	0.38
Truxton	6.90	0.85	3.17	0.67	0.28	0.27	6.75	0.81	2.17	0.90	1.21	0.54
Cicero	6.64	1.05	3.99	0.85	0	.	6.48	1.01	3.22	1.04	1.05	0.69
Owasco	6.63	0.70	2.66	0.55	0	.	6.65	0.72	1.99	0.78	0.21	0.32
Sherburne	6.44	0.82	3.12	0.62	0	.	6.45	0.82	2.41	0.86	0.81	0.40
SV1	6.40	0.89	3.01	0.75	2.58	1.26	6.31	0.77	2.21	0.91	-0.70	0.48
94001	6.16	0.77	2.70	0.62	0.66	0.57	6.17	0.77	2.24	0.82	-0.19	0.38
Onondaga	5.98	0.72	2.73	0.56	0	.	5.96	0.78	2.25	0.83	-0.52	0.35
Allegany	5.96	0.69	2.61	0.53	0	.	6.02	0.74	2.09	0.77	0	.
Wolcott	5.84	0.87	2.98	0.81	0.94	0.96	6.07	0.86	2.61	0.90	-0.02	0.57
S25	4.96	0.65	2.24	0.52	0.45	0.45	4.91	0.63	1.36	0.76	0.39	0.40
							σ^2_{ENV}		3.66	2.31		
							σ^2_{β}		0.06	0.05		
	σ^2_{BLK}		0.91	0.24			σ^2_{BLK}		0.90	0.23		

Table 3.6
(continued)

σ^2_{RESj}	Albion	0.99	0.19	σ^2_{RESj}	Albion	0.92	0.18
	Bellev	3.92	0.88		Bellev	1.86	0.39
	Boisbr	5.84	1.00		Boisbr	6.02	1.01
	Brimle	0.41	0.08		Brimle	0.43	0.08
	Consta	4.14	0.73		Consta	4.53	0.79
	Escana	2.17	0.45		Escana	2.20	0.46
	Fredon	1.38	0.24		Fredon	1.35	0.25
	Middle	3.65	0.66		Middle	3.73	0.81
	Postdam	1.05	0.22		Postdam	1.20	0.26
	Saskat	1.08	0.20		Saskat	1.12	0.21
	Savoy	2.98	0.67		Savoy	3.36	0.72
	Skandi	0.65	0.12		Skandi	0.67	0.12
	Storrs	6.81	1.29		Storrs	6.66	1.28
	Tully	0.66	0.13		Tully	0.72	0.15
	Waseca	1.48	0.25		Waseca	2.03	0.39

The partial least squares regression analysis for Dataset 1 reflected the broad geographic variability across trial locations (Figure 3.2). The first factor showed a pattern of increasing temperatures and growing degree days at lower latitudes. Since growing season maximum temperature (Tmax), growing degree days (GDD) and annual minimum temperature (Tmin) are correlated with one another, these factors all group together on the right-hand side of the biplot.

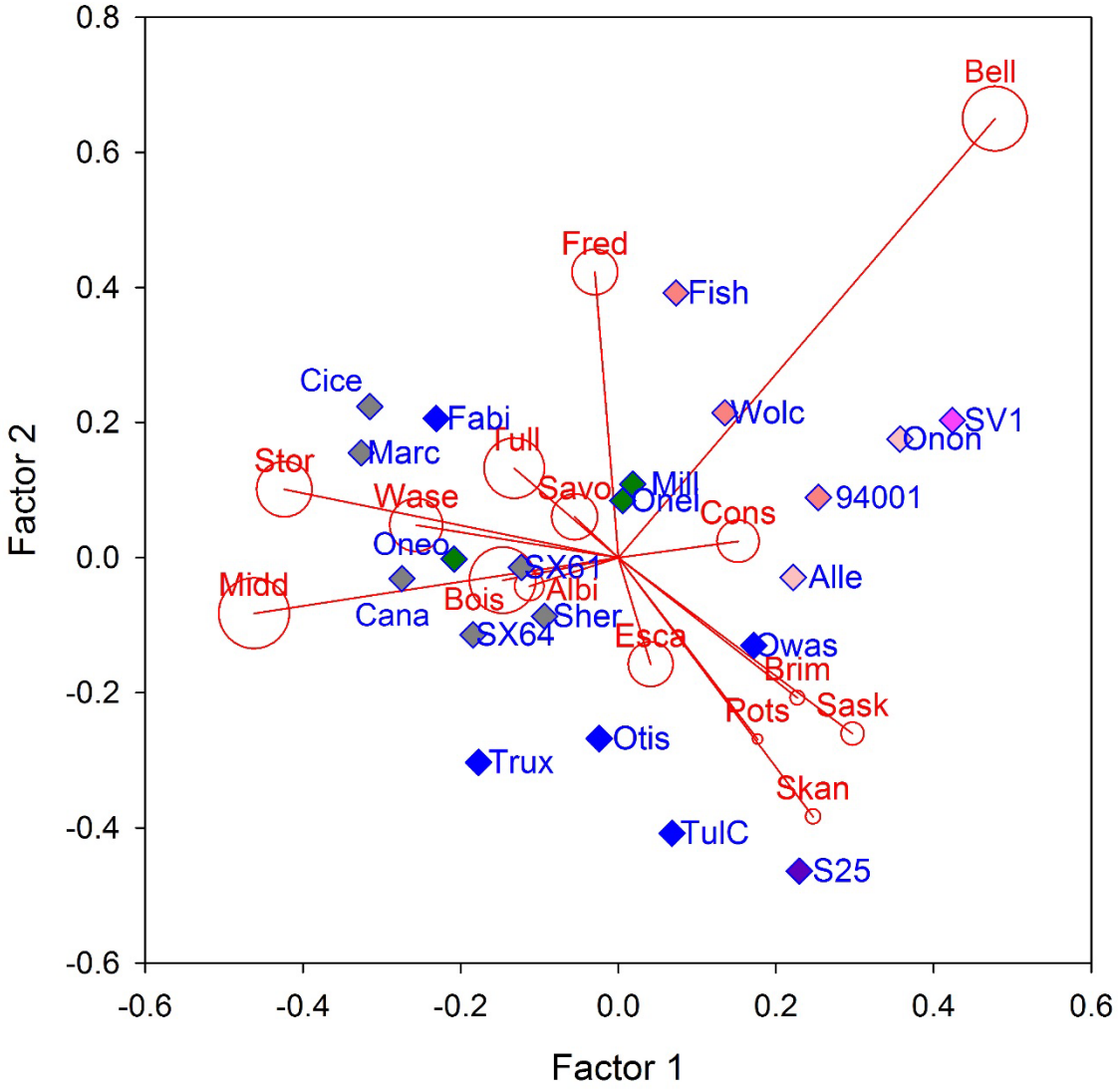


Figure 3.1 Mixed model AMMI-2 biplot for Dataset 1, representing the first two factor scores for environments (red) and genotypes (blue), with names truncated.

Tetraploid *S. miyabeana* genotypes such as ‘Marcy’, ‘Cicero’, ‘Canastota’ and ‘SX64’together with the top-yielding *S. viminalis* × *S. miyabeana* triploid hybrid, ‘Fabius’, grouped with these temperature-related variables, suggesting better performance in warmer environments. In contrast, ‘Tully Champion’, also a *S. viminalis* × *S. miyabeana* triploid grouped with the variable latitude (LAT) with was on the opposite end of the projection of factor 1, suggesting this cultivar performed better in cooler environments. Factor two seemed to be associated with increasing precipitation and decreasing solar radiation along a longitudinal gradient. The *S. viminalis* × *S. miyabeana* cultivar ‘Otisco’ and the *S. purpurea* ‘Fish Creek’ had strong, but opposite loadings along the second PLS factor. However, it should be noted that in this analysis only about 25% of the overall variation in interactions could be explained by the first two PLS factors.

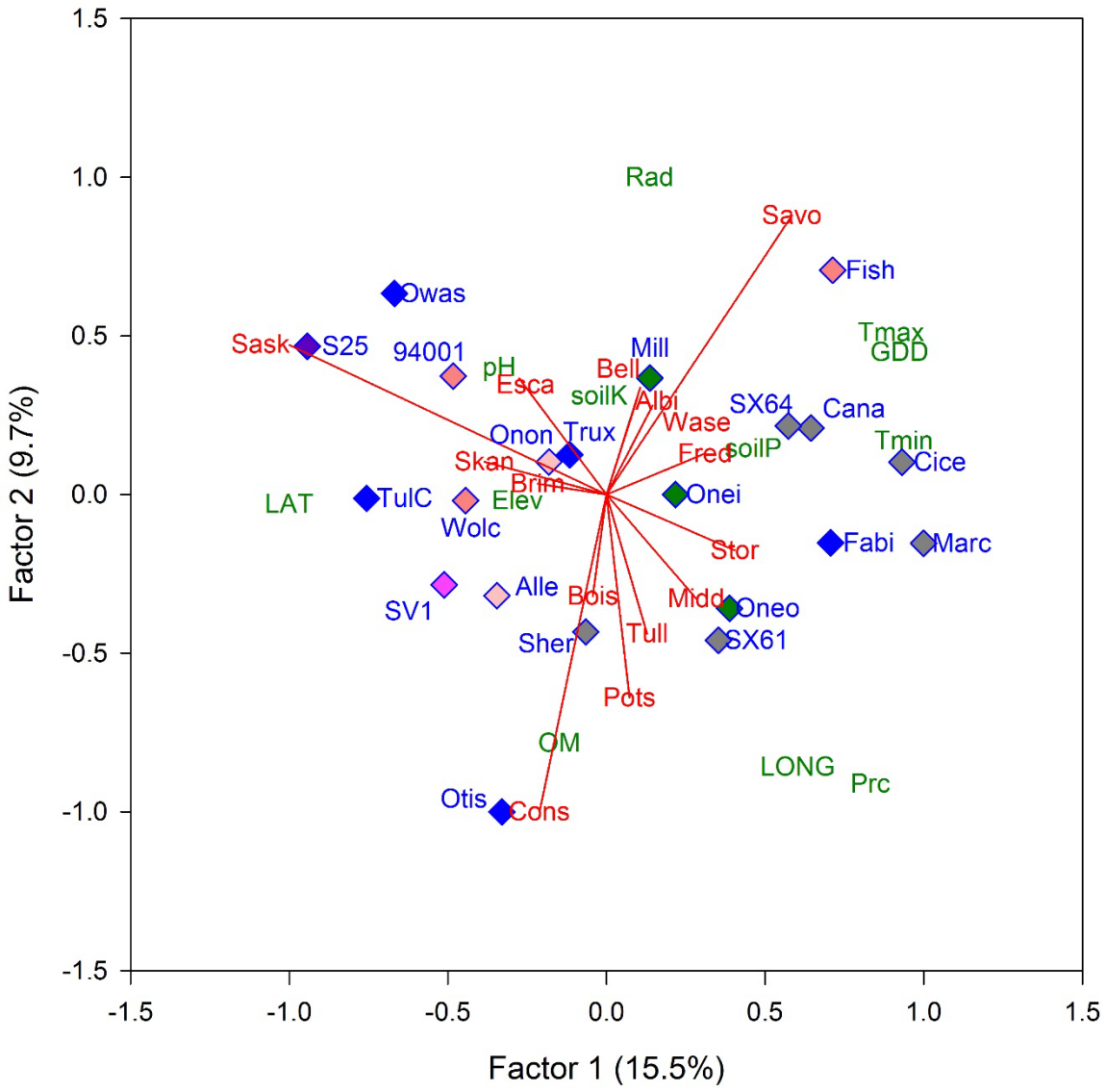


Figure 3.2 Partial least squares biplot for Dataset 1, representing the first two factors for environment scores (red), and genotype (blue) and environmental covariate (green) loadings, with environment and genotype names truncated.

3.4.2 Yield Trial Dataset 2

The same mixed modeling approach described above was used to analyze 25 genotypes planted in five environments, consisting of new selections and some commercial cultivars also present in Dataset 1 (Table 3.7). The AMMI-2 and E-R models again provided the best model fit

for the case of homogeneous error variance. Again, log likelihood ratio tests showed that heterogeneous error variance provided a better fit in all cases. Based on -2 Res LL, AMMI-2 and E-R models were the best models, and based on AIC, AMMI-2 and AMMI-1 models had the best fits, despite AMMI-2 having the greatest number of model parameters to be estimated. There were some contrasting differences for variance parameters between AMMI-1 and AMMI-2 models (Table 3.8). Most notably, the main effect of environment was estimated at zero for the AMMI-2 model, as was the common residual variance, σ^2_β, present in the FA1 variance-covariance structure. This is likely due to the relatively low number of testing environments. In light of this, the more parsimonious AMMI-1 model is likely a better fit for Dataset 2.

Table 3.7 Fit statistics for mixed models tested in Dataset 2.

	Homogeneous Error Variance			Heterogeneous Error Variance			Res LL[a] Ratio Tests		
Model	***p*[b]**	**-2 Res LL**	**AIC[c]**	***p***	**-2 Res LL**	**AIC**	***X*²**	**DF**	***P*-value**
Simple additive	3	1908.6	1914.6	7	1770.6	1784.6	138	4	<0.0001
Compound Symmetry	4	1870.1	1878.1	8	1743.4	1759.4	126.7	4	<0.0001
Heterogeneous CS	28	1812.6	1868.6	32	1666.7	1728.7	145.9	4	<0.0001
Shukla's	28	1844.8	1882.8	32	1714	1764	130.8	4	<0.0001
Finlay-Wilkinson	28	1815.7	1871.7	32	1666.7	1730.7	149	4	<0.0001
Eberhart-Russell	52	**1793.9**	**1869.9**	56	**1654.3**	1738.3	139.6	4	<0.0001
AMMI-1	29	1815.7	1873.7	33	1665.9	**1731.9**	149.8	4	<0.0001
AMMI-2	53	**1756.9**	**1856.9**	57	**1617.7**	**1725.7**	139.2	4	<0.0001

[a]Res LL, Residual log likelihood ratio tests, where the Chi-squared statistic is the difference in the -2 log likelihood, and degrees of freedom (DF) is the difference in the number of parameters, between homogeneous and heterogeneous cases.
[b]Number of parameters estimated in the variance-covariance for each model including the effect of block (nested within environment).
[c]AIC, Akaike information criterion. Bold and underlined values of -2 Res LL and AIC indicate best model fit.

Table 3.8 Variance parameters for best fitting models under heterogeneous residual error variance according to AIC for Dataset 2.

	AMMI-1				AMMI-2					
Genotype	**LS Mean**	**SE**	**$\lambda_{i,1}$**	**SE**	**LS Mean**	**SE**	**$\lambda_{i,1}$**	**SE**	**$\lambda_{i,2}$**	**SE**
LA970253	8.31	1.99	4.64	1.78	6.96	1.50	1.90	2.20	3.46	1.57
Preble	8.15	2.04	4.79	1.77	8.10	2.12	5.04	1.84	0.32	0.66
Tully Champion	8.06	1.34	3.06	2.57	10.46	0.43	0.13	2.29	-1.18	1.30
Tonawanda	8.05	1.87	4.37	1.63	8.00	1.92	4.55	1.68	0.38	0.64
Victor	7.77	1.92	4.48	1.67	7.91	1.84	3.97	1.82	2.09	0.93
Fabius	7.73	1.53	3.53	1.36	7.71	1.59	3.75	1.40	0.28	0.59
Saratoga	7.31	1.69	3.93	1.49	7.38	1.63	3.58	1.57	1.62	0.79
Sterling	7.01	1.37	3.07	1.28	9.13	2.11	3.73	2.29	3.36	2.43
Hopewell	6.97	1.50	3.43	1.40	9.49	2.34	4.23	2.64	3.97	2.56
Millbrook	6.90	1.55	3.58	1.38	6.91	1.54	3.54	1.40	0.92	0.62
Geneva Giant	6.85	1.73	4.03	1.53	7.00	1.68	3.48	1.71	2.17	0.93
Seneca Chief	6.78	1.40	3.20	1.26	6.69	1.52	3.54	1.33	0	.
Otisco	6.69	1.50	3.45	1.33	6.63	1.58	3.73	1.39	0.09	0.55
SX61	6.53	1.66	3.86	1.46	6.48	1.70	3.99	1.49	0.46	0.58
02X-326-015	6.05	1.35	3.09	1.22	5.94	1.38	3.23	1.23	0.20	0.53
05X-291-050	5.82	1.17	2.65	1.09	5.81	1.18	2.68	1.10	0.66	0.55
Sheridan	5.71	1.30	2.98	1.18	5.69	1.31	3.02	1.19	0.59	0.54
01X-265-019	5.58	1.26	2.85	1.14	5.49	1.33	3.10	1.17	0	.
Fish Creek	5.58	0.98	2.15	0.94	5.65	0.97	2.03	1.00	1.05	0.60
02X-326-010	5.53	1.18	2.67	1.09	5.35	1.39	3.19	1.26	-0.68	0.56
Canastota	5.34	2.39	5.61	2.93	5.66	2.20	5.19	2.86	1.04	1.33
Dimitrios	4.99	0.68	1.24	0.71	5.00	0.64	0.99	0.78	1.04	0.59
94006	4.73	1.03	2.29	0.98	4.71	1.00	2.18	0.98	0.84	0.57
05X-293-047	4.57	0.77	1.44	0.84	5.99	1.31	1.92	1.50	2.25	1.95
India	3.14	0.67	1.26	0.68	3.12	0.61	1.23	0.66	0.50	0.48

Table 3.8
(continued)

σ^2_{ENV}		0.36	0.67	σ^2_{ENV}	0	.
σ^2_{β}		0.002	0.06	σ^2_{β}	0	.
σ^2_{BLK}		0.43	0.21	σ^2_{BLK}	0.45	0.20
$\sigma^2_{RES_j}$	Escanaba	5.26	0.95	$\sigma^2_{RES_j}$	4.91	0.83
	Fredonia, Amended	1.10	0.21		1.06	0.18
	Fredonia, Control	0.39	0.07		0.37	0.06
	Geneva	4.88	0.87		3.96	0.62
	Rock Springs	4.35	0.81		2.99	0.51

A mixed model version of an AMMI-1 biplot was constructed using the first factor scores and model yield estimates for genotypes (Fig. 3.3). Four genotypes, LA970253, ‘Preble’, ‘Tonawanda’ and ‘Victor’ produced greater or similar yields compared to ‘Fabius’ and ‘Tully Champion’, which were identified as having high yields and specific adaptability in Dataset 1. LA970253 and ‘Preble’ are *S. viminalis* × *S. miyabeana* triploids, whereas ‘Tonawanda’ is a (*S. koriyanagi* × *S. purpurea*) × *S. miyabeana* triploid, and ‘Victor’ is a *S. purpurea* × *S. miyabeana* triploid. These four new cultivars had greater than average factor 1 scores and the linear nature of the relationship between yield and factor 1 scores suggests that these genotypes are well adapted to higher quality environments. In addition to those top-performing genotypes, an additional five previously untested genotypes had mean yields greater than the check cultivar, ‘SX61’ and near average interaction scores (Fig. 3.3). Cultivars ‘India’ and ‘Dimitrios’ are from Canadian and Swedish breeding programs, respectively, and performed poorly in our trials. ‘Dimitrios’ was frequently observed to show signs of mammalian browsing and ‘India’ was highly susceptible to potato leafhopper (*Empoasca fabae* Harris), a known pest for some species of shrub willow. Three *S. purpurea* genotypes, 05X-293-047, 94006 and ‘Fish Creek’ also grouped together with low yields and below average interaction variance parameters.

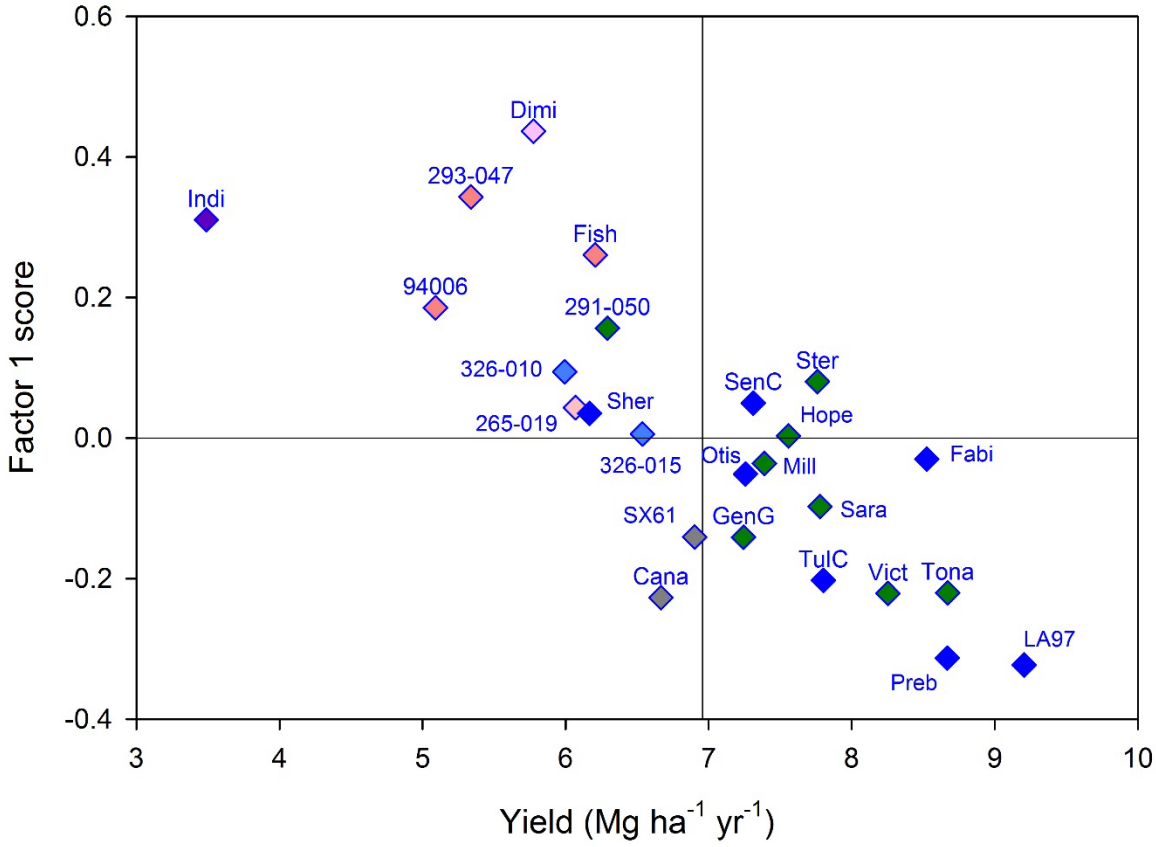


Figure 3.3 Mixed model AMMI-1 biplot showing genotype least square means for yield and first factor scores for Dataset 2. Genotype names have been truncated. Vertical and horizontal reference lines represent the mean yield and mean factor score, respectively.

The PLS analysis for Dataset 2 indicated differences across a latitudinal gradient, with the southernmost location, Rock Springs, PA, being associated with higher temperatures, as well as greater growing season precipitation for factor 1 compared to the northernmost location, Escanaba, MI, which was also associated with higher soil pH and phosphorus levels (Fig. 3.4). A number of the *S. viminalis* × *S. miyabeana* triploids grouped on the right-hand side of the figure indicating a positive association with higher temperatures. Factor 2 seemed to be associated with differences in soil potassium levels between Geneva and the Fredonia Amended trials. The

Fredonia Amended trial had the highest potassium levels, and all of the *S. purpurea* genotypes grouped with that trial, suggesting a positive response to greater soil fertility for that species.

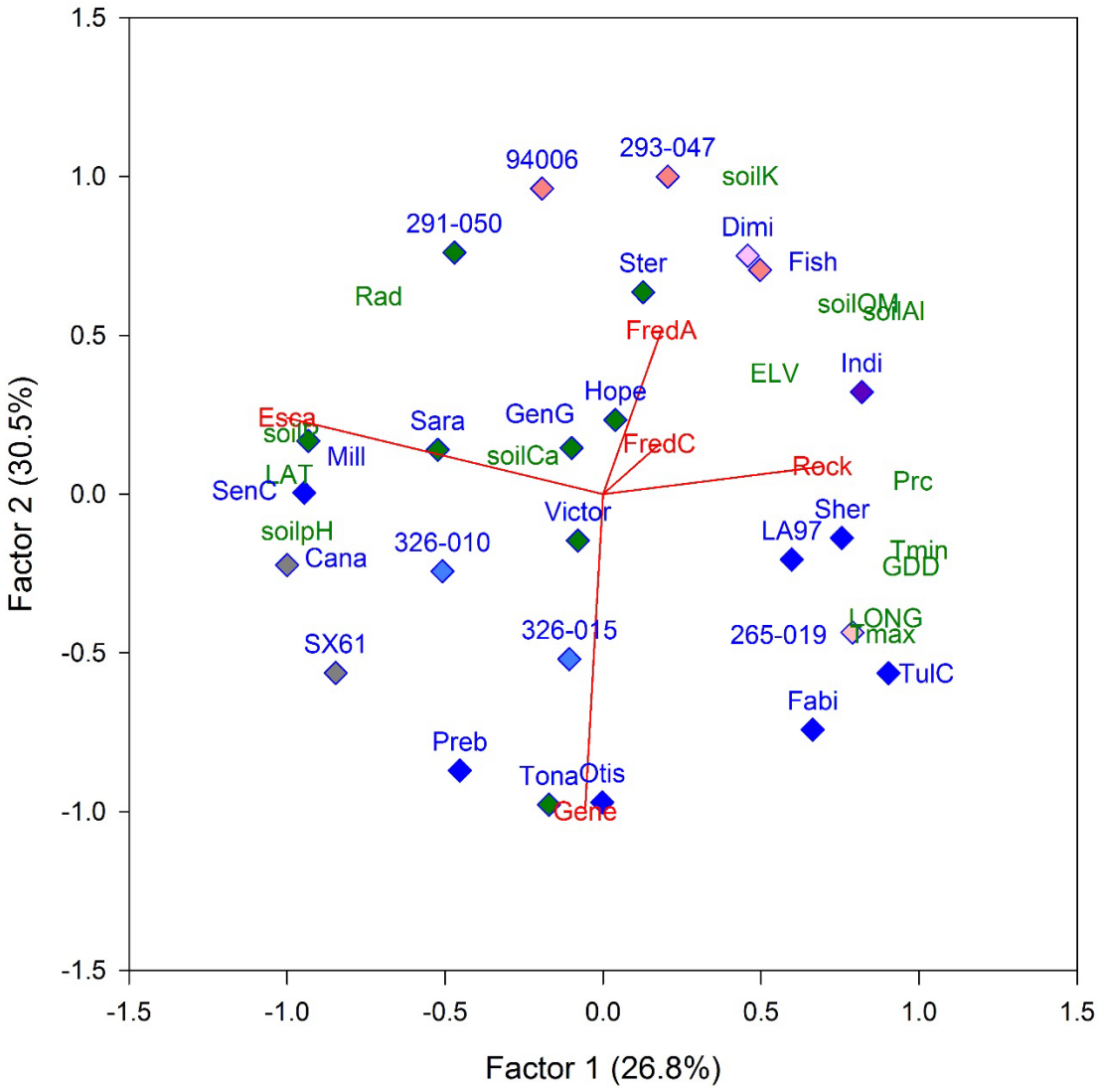


Figure 3.4 Partial least squares biplot for Dataset 2, representing the first two factors for environment scores (red), and genotype (blue) and environmental covariate (green) loadings, with environment and genotype names truncated.

3.5 Discussion

In our analysis we have demonstrated that breeding efforts have made substantial gains in yield over the foundational material, which served as checks in these newer evaluations. In a previous fixed effects AMMI analysis of a smaller dataset (Fabio et al 2016), 'Fabius' and 'Tully Champion' were found to be top-performing cultivars in contrasting high and low-yielding environments, respectively (Fabio et al. 2017). The present expanded mixed model analysis was conducted over a larger number of environments and included a larger number of cultivars, and we confirmed the superior performance of 'Fabius', with a 14% increase in overall yield compared to the mean of the check cultivars. 'Tully Champion', however, showed a yield advantage in the environments with the lowest overall yields, suggesting specific adaptation. Our expanded analysis also highlighted other promising cultivars from other pedigrees, such as 'Oneonta', a *S. purpurea* × *S. miyabeana* triploid, and 'Marcy', an improved *S. miyabeana* tetraploid, thus diversifying the array of commercially available cultivars.

Genotype-by-environment interactions in shrub willow have been noted in European studies (Larsen et al. 2014; Rönnberg-Wästljung and Thorsén 1988). However, until recently, data on cultivar performance from multiple shrub willow yield trials was limited for North America, largely due to the longer generation times and production cycles for this perennial crop. In some early work on cultivar evaluations, Kiernan et al. (2003) tested foundational germplasm prior to any US breeding improvements and found that 'SX61' and 'SX64' exhibited both high and stable yields.

Dataset 2 provided an opportunity to test new breeding material against cultivars identified in Dataset 1 as high-yielding and specifically adapted to both low and high quality environments. Again, incorporating heterogeneous error variances significantly improved model

fit. AMMI models provided the best fit, but a number of variance estimates were estimated at zero for the AMMI-2 model, including the main effect of environment, suggesting this model was not well suited for this smaller dataset containing only five environments. We selected the more parsimonious AMMI-1 as the best representation of Dataset 2, but it should be recognized that standard errors of interaction variance estimates were still rather large for this dataset (Table 3.6). Patterns in yield and adaptability exhibited by 'Fabius' and 'Tully Champion' in Dataset 2 were similar to those of Dataset 1, in that both cultivars maintained high yields, but had contrasting interaction variances. In Dataset 2, a new group of genotypes emerged as promising, including two *S. viminalis* × *S. miyabeana* triploids, LA970253 and 'Preble'. These two new selections are of the same diversity group as 'Fabius' and 'Tully Champion' and had on average 4.2% greater yields over those cultivars and 26% greater yields over the commercial check, 'SX61'. Triploids 'Tonawanda' ((*S. koriyanagi* × *S. purpurea*) × *S. miyabeana*) and 'Victor' (*S. purpurea* × *S. miyabeana*) had on average a 14.6% yield advantage over the check within the same diversity, 'Millbrook', and a 21.1% increase over 'SX61'. Within the *S. purpurea* diversity group, we did not observe new genotypes with yield increases over the commercial cultivar 'Fish Creek'. However one genotype, 01X-265-019 (*S. koriyanagi* × *S. purpurea*) had an equivalent mean yield compared to 'Fish Creek', whereas in Dataset 1, other *S. koriyanagi* × *S. purpurea* genotypes had mean yields 16.9% lower than 'Fish Creek'.

The breeding history of shrub willow as a dedicated bioenergy crop in North America is brief, but employing a strategy of developing interspecific hybrids displaying heterosis has shown potential for improving yields in early selection trials for this emerging crop (Serapiglia et al. 2014). These interspecific hybrids have simultaneously allowed for substantial yield gains and integrated resistance to pests and diseases. Improved *S. viminalis* and associated interspecific

hybrids in Europe has led to greater yields (Aylott et al. 2008; Larsen et al. 2014; Lindegaard et al. 2011); however, it was recognized early on that *S. viminalis* cultivars in the US were highly susceptible to potato leafhopper (Labrecque and Teodorescu 2005; Smart and Cameron 2008). In the US breeding program, hybridization of *S. viminalis* with *S. miyabeana* has produced some of the greatest gains in yield, as demonstrated by 'Fabius' and 'Tully Champion' in Dataset 1. These two cultivars are siblings, but interestingly have been shown to have varying degrees of susceptibility to potato leafhopper (Gouker and Smart 2015). In our analysis of Dataset 2 'Preble', a cross between the same *S. viminalis* mother and an improved *S. miyabeana*, was shown to outperform 'Fabius' and 'Tully Champion'.

In further support of yield improvements through interspecific hybridization, new triploid selections such as 'Tonawanda' ((*S. koriyanagi* × *S. purpurea*) × *S. miyabeana*) and 'Victor' (*S. purpurea* × *S. miyabeana*) outperformed both improved *S. purpurea* and *S. miyabeana* cultivars, and performed similarly to 'Fabius' and 'Tully Champion'. These genotypes have not demonstrated susceptibility to common willow pests, and perhaps more importantly they appear to be more resistant to leaf rust (*Melampsora* spp), which can be damaging to pure *S. purpurea*. Since pest and disease populations can evolve rapidly, deployment of diverse cultivars in future commercial plantings will likely provide a safeguard against potential pest and disease outbreaks. For instance, research in the UK has shown that planting polycultures of diverse cultivars can maintain greater yields over monocultures of *Salix* spp. under pressure from rust (McCracken and Dawson 2003; McCracken et al. 2011).

In a similar mixed model analysis of numerous interspecific poplar (*Populus*) hybrids grown in Spain for biomass production, Sixto et al. (2014) also found evidence of differences in genotypic adaptations among different taxonomic groups. They argue that adaptive patterns may

have been influenced by the geographic origin (i.e. Europe or North America) of the parental species and that hybrids involving the European native *P. nigra* may possess specific adaptation to the Mediterranean climate. In our analysis we did not find evidence supporting the notion that native species or hybrids involving natives are better adapted when grown in North America. *Salix eriocephala* is a native species of North America and cultivar 'S25' in Dataset 1 was the lowest yielding cultivar overall. Interspecific hybridization involving *S. eriocephala* has proven to be difficult (Kopp et al. 2001), and intraspecific breeding has not been shown to improve rust resistance in F_1 progeny (Cameron et al. 2008). *S. purpurea* is a naturalized species of North America, and both natural accessions and improved cultivars showed poor performance in general across both of our datasets. Both *S. eriocephala* and *S. purpurea* are susceptible to leaf rust, which may have contributed to lower yields. Current efforts are underway to characterize the pathogen-host relationship between rust and *S. purpurea* and to evaluate novel interspecific F_1 families for improved rust resistance.

Sixto et al. (2014) also speculated that a three-way hybrid involving *P. nigra* performed worse than the two-way hybrid, which the authors attribute to a possible reduction in the genetic contribution of *P. nigra* to biomass traits in the three-way cross, leading to poorer performance. In our Dataset 2, we tested three three-way hybrids, 05X-326-010, 05X-326-015 and 'Dimitrios', which involved a *S. viminalis* × *S. schwerinii* parent, and they all exhibited poor performance, with low mean yields and below average interaction variance parameters. The apparent superior biomass traits possessed by *S. viminalis* could have been diminished in a similar way in this complex cross, although in the case of 'Dimitrios', poor performance was likely due to increased pest pressure based on visual surveys in the field.

The use of partial least squares regression allowed us to test for relationships between GEI and environmental covariates. The geographic range among the test environments was relatively large, and latitudinal and longitudinal gradients were apparent, although only about 25% of the total variation in GEI response was explained by PLS in Dataset 1. Some of the top-performing cultivars such as 'Fabius' and 'Marcy' were positively associated with higher temperatures and greater growing degree days (Figure 3.3). Sixto et al. (2015) used factorial regression and found differential responses of poplar hybrids to latitude and maximum temperatures during the growing season. For Dataset 2, the smaller number of environments covered a relatively smaller geographic range, but we were able to capture more than twice the variability in genotype response variables compared to Dataset 1. We had more complete records for soil nutrient concentrations in Dataset 2 and some of the patterns revealed through PLS suggest some genotypic differences in response to particular nutrients, such as phosphorus and potassium, but further testing is needed in this area. Some of the new triploid hybrids in Dataset 2 were also positively associated with higher temperatures and growing degree days as they were in Dataset 1. A proposed production zone of shrub willow in the US was previously restricted to more northern latitudes (Walsh et al. 2003), but our results suggest that a southward expansion of this boundary should be tested, perhaps particularly in the case of the triploid hybrids. This could have implications for models that compare production potential of willow and C_4 perennials like switchgrass (*Panicum virgatum* L.) and Miscanthus across large geographic and climatic regions.

For a perennial crop like shrub willow, the term yield stability has an additional dimension in the sense that a single crop is proposed to have a productive life of over 25 years, or for at least seven three-year harvest cycles. While we have reported yield for only the first harvest cycle, Sleight et al. (2016) analyzed first and second rotation data from a subset of the

yield trials in Dataset 1 and found a strong relationship in first rotation yields determining the relative change in yields of the second rotation. They proposed that poor establishment leads to low first rotation yields, but those poor performing trials tend to have the greatest relative increase in yield in the second rotation. A follow up analysis of data across three harvest cycles at two locations showed little change in rank among cultivar yields (Sleight and Volk 2016). A truly accurate assessment of shrub willow cultivar stability and adaptability will likely need to involve multiple rotations, and a mixed model framework as proposed earlier has (Piepho and Eckl 2014) and used here.

3.6 Conclusion

The use of mixed models has allowed us to analyze a large yet unbalanced dataset of shrub willow yields in order to characterize GEI. The main findings are incremental increases in yield were achieved through traditional breeding techniques, that interspecific triploid hybrids seem to express the largest yield gains, especially in warmer environments, and that the GEI allows for the identification of cultivars adapted to low and high yielding environments. This is critical for deploying regionally adapted and high yielding genotypes for stands expected to be productive over 20 years. As a result of this work we have identified a number of recently bred, high-yielding triploid hybrids from diverse pedigrees that will be advanced for commercialization. Recurrent selection of improved of diploid and tetraploid progeny for the development of new triploid hybrids will be a key focus of future breeding efforts.

3.7 References

Aronsson, P., Rosenqvist, H., Dimitriou, I. (2014) Impact of Nitrogen Fertilization to Short-Rotation Willow Coppice Plantations Grown in Sweden on Yield and Economy. Bioenerg. Res. 7, 993-1001.

Aylott, M.J., Casella, E., Tubby, I., Street, N.R., Smith, P., Taylor, G. (2008) Yield and spatial supply of bioenergy poplar and willow short-rotation coppice in the UK. New Phytologist 178, 358-370.

Buchholz, T., Volk, T.A. (2011) Improving the profitability of willow crops-identifying opportunities with a crop budget model. Bioenerg. Res. 4, 85-95.

Cameron, K.D., Phillips, I.S., Kopp, R.F., Volk, T.A., Maynard, C.A., Abrahamson, L.P., Smart, L.B. (2008) Quantitative genetics of traits indicative of biomass production and heterosis in 34 full-sib F_1 *Salix eriocephala* families. Bioenerg. Res. 1, 80-90.

Caputo, J., Balogh, S.B., Volk, T.A., Johnson, L., Puettmann, M., Lippke, B., O'Neil, E. (2014) Incorporating uncertainty into a life cycle assessment (LCA) model of short-rotation willow biomass (*Salix* spp.) crops. Bioenerg. Res. 7, 48-59.

Crossa, J., Vargas, M., Cossani, C.M., Alvarado, G., Burgueño, J., Mathews, K.L., Reynolds, M.P. (2015) Evaluation and interpretation of interactions. Agron. J. 107, 736-747.

Cunniff, J., Purdy, S.J., Barraclough, T.J.P., Castle, M., Maddison, A.L., Jones, L.E., Shield, I.F., Gregory, A.S., Karp, A. (2015) High yielding biomass genotypes of willow (*Salix* spp.) show differences in below ground biomass allocation. Biomass Bioenerg. 80, 114-127.

Denis, J.B., Piepho, H.P., VanEeuwijk, F.A. (1997) Modelling expectation and variance for genotype by environment data. Heredity 79, 162-171.

Djomo, S.N., Kasmioui, O.E., Ceulemans, R. (2011) Energy and greenhouse gas balance of bioenergy production from poplar and willow: a review. GCB Bioenergy 3, 181-197.

Djomo, S.N., Witters, N., Van Dael, M., Gabrielle, B., Ceulemans, R. (2015) Impact of feedstock, land use change, and soil organic carbon on energy and greenhouse gas performance of biomass cogeneration technologies. Appl. Energy 154, 122-130.

Eberhart, S.A., Russell, W.A. (1966) Stability parameters for comparing varieties. Crop Sci. 6, 36-40.

Environment Canada (2015) National Climate Data. http://climate.weather.gc.ca/. (accessed 16.05.16).

Environmental Protection Agency (2007) Finalized Regulations for a Renewable Fuel Standard (RFS) Program for 2007 and Beyond. 72 Fed. Reg. 23900.

Fabio, E.S., Volk, T.A., Miller, R.O., Serapiglia, M.J., Gauch, H.G., Van Rees, K.C.J., Hangs, R.D., Amichev, B.Y., Kuzovkina, Y.A., Labrecque, M., Johnson, G.A., Ewy, R.G., Kling, G.J., Smart, L.B. (2017) Genotype × environment interaction analysis of North American shrub willow yield trials confirms superior performance of triploid hybrids. GCB Bioenergy 9:445-459.

Finlay, K.W., Wilkinson, G.N. (1963) The analysis of adaptation in a plant-breeding programme. Crop and Pasture Science 14, 742-754.

Gauch, H.G. (1988) Model selection and validation for yield trials with interaction. Biometrics 44, 705-715.

Gollob, H.F. (1968) A statistical model which combines features of factor analytic and analysis of variance techniques. Psychometrika 33, 73-115.

Gouker, F.E., Smart, L.B., 2015. Genetic Diversity of Willow fact sheet. http://willow.cals.cornell.edu/. (accessed 17.8.16).

Hangs, R.D., Schoenau, J.J., Van Rees, K.C.J., Bélanger, N., Volk, T., Jensen, T. (2014) First rotation biomass production and nutrient cycling within short-rotation coppice willow plantations in Saskatchewan, Canada. Bioenerg. Res. 7, 1091-1111.

Hauk, S., Knoke, T., Wittkopf, S. (2014) Economic evaluation of short rotation coppice systems for energy from biomass—A review. Renewable and Sustainable Energy Reviews 29, 435-448.

Hu, X.Y. (2014) Combined yield comparison and stability evaluation of rape genotypes using the mixed model. Field Crop Res 167, 11-18.

Hu, X.Y., Spilke, J. (2011) Variance-covariance structure and its influence on variety assessment in regional crop trials. Field Crop Res 120, 1-8.

Kiernan, B.D., Volk, T.A., Tharakan, P.J., Nowak, C.A., Phillipon, S.P., Abrahamson, L.P., White, E.H. (2003) Clone-site testing and selections for scale-up plantings, Final Report prepared for the United States Department of Energy. SUNY-ESF, Syracuse, NY, p. 67 pp.

Kopp, R.F., Smart, L.B., Maynard, C.A., Isebrands, J.G., Tuskan, G.A., Abrahamson, L.P. (2001) The development of improved willow clones for eastern North America. For. Chron. 77, 287-292.

Kumar, A., Verulkar, S.B., Mandal, N.P., Variar, M., Shukla, V.D., Dwivedi, J.L., Singh, B.N., Singh, O.N., Swain, P., Mall, A.K., Robin, S., Chandrababu, R., Jain, A., Haefele, S.M., Piepho, H.P., Raman, A. (2012) High-yielding, drought-tolerant, stable rice genotypes for the shallow rainfed lowland drought-prone ecosystem. Field Crop Res 133, 37-47.

Kuzovkina, Y.A., Quigley, M.F. (2005) Willows beyond wetlands: Uses of *Salix* L. species for environmental projects. Water Air Soil Pollut. 162, 183-204.

Labrecque, M., Teodorescu, T.I. (2005) Field performance and biomass production of 12 willow and poplar clones in short-rotation coppice in southern Quebec (Canada). Biomass Bioenerg. 29, 1-9.

Larocque, G.R., DesRochers, A., Larchevêque, M., Tremblay, F., Beaulieu, J., Mosseler, A., Major, J.E., Gaussiran, S., Thomas, B.R., Sidders, D., Périnet, P., Kort, J., Labrecque, M., Savoie, P., Masse, S., Bouman, O.T., Kamelchuk, D., Benomar, L., Mamashita, T., Gagné, P. (2013) Research on hybrid poplars and willow species for fast-growing tree plantations: Its importance for growth and yield, silviculture, policy-making and commercial applications. For. Chron. 89, 32-41.

Larsen, S.U., Jørgensen, U., Lærke, P.E. (2014) Willow yield is highly dependent on clone and site. Bioenerg. Res. 7, 1280-1292.

Lindegaard, K.N., Carter, M.M., McCracken, A., Shield, I.F., MacAlpine, W., Jones, M.H., Valentine, J., Larsson, S. (2011) Comparative trials of elite Swedish and UK biomass willow varieties 2001-2010. Asp. Appl. Biol. 112, 57-66.

McCracken, A.R., Dawson, W.M. (2003) Rust disease (*Melampsora epitea*) of willow (*Salix* spp.) grown as short rotation coppice (SRC) in inter- and intra-species mixtures. Ann. Appl. Biol. 143, 381-393.

McCracken, A.R., Walsh, L., Moore, P.J., Lynch, M., Cowan, P., Dawson, M., Watson, S. (2011) Yield of willow (*Salix* spp.) grown in short rotation coppice mixtures in a long-term trial. Ann. Appl. Biol. 159, 229-243.

McKenney, D.W., Weersink, A., Allen, D., Yemshanov, D., Boyland, M. (2014) Enhancing the adoption of short rotation woody crops for bioenergy production. Biomass Bioenerg. 64, 363-366.

Mosseler, A., Major, J.E., Labrecque, M. (2014) Genetic by environment interactions of two North American Salix species assessed for coppice yield and components of growth on three sites of varying quality. Trees 28, 1401-1411.

NASA POWER (2015) Prediction of worldwide energy resource climatology resource for agroclimatology. http://power.larc.nasa.gov/. (accessed 16.05.16).

NOAA NCEI (2015) Global historical climatology network. http://www.ncdc.noaa.gov/data-access. (accessed 16.05.16).

Pacaldo, R., Volk, T., Briggs, R. (2013) Greenhouse gas potentials of shrub willow biomass crops based on below- and aboveground biomass inventory along a 19-year chronosequence. Bioenerg. Res. 6, 252-262.

Pacaldo, R., Volk, T., Briggs, R. (2014) Carbon sequestration in fine roots and foliage biomass offsets soil CO_2 effluxes along a 19-year chronosequence of shrub willow (*Salix* x *dasyclados*) biomass crops. Bioenerg. Res. 7, 769-776.

Piepho, H.P. (1997) Analyzing genotype-environment data by mixed models with multiplicative terms. Biometrics 53, 761-766.

Piepho, H.P. (1998) Methods for comparing the yield stability of cropping systems. J. Agron. Crop Sci. 180, 193-213.

Piepho, H.P. (1999) Stability analysis using the SAS system. Agronomy Journal 91, 154-160.

Piepho, H.P., Büchse, A., Emrich, K. (2003) A Hitchhiker's Guide to Mixed Models for Randomized Experiments. J. Agron. Crop Sci. 189, 310-322.

Piepho, H.P., Eckl, T. (2014) Analysis of series of variety trials with perennial crops. Grass Forage Sci. 69, 431-440.

Raman, A., Ladha, J.K., Kumar, V., Sharma, S., Piepho, H.P. (2011) Stability analysis of farmer participatory trials for conservation agriculture using mixed models. Field Crop Res. 121, 450-459.

Rönnberg-Wästljung, A., Thorsén, J. (1988) Inter- and intraspecific variation and genotype × site interaction in *Salix alba* L., *S. dasyclados* Wimm. and *S. viminalis* L. Scand. J. For. Res. 3, 449-463.

SAS Institute Inc. (2013) 9.4 ed. SAS Institute, Inc, Cary, NC, USA.

Serapiglia, M.J., Cameron, K.D., Stipanovic, A.J., Abrahamson, L.P., Volk, T.A., Smart, L.B. (2013) Yield and woody biomass traits of novel shrub willow hybrids at two contrasting sites. Bioenerg. Res. 6, 533-546.

Serapiglia, M.J., Gouker, F.E., Hart, J.F., Unda, F., Mansfield, S.D., Stipanovic, A.J., Smart, L.B. (2015) Ploidy level affects important biomass traits of novel shrub willow (*Salix*) hybrids. Bioenerg. Res. 8, 259-269.

Serapiglia, M.J., Gouker, F.E., Smart, L.B. (2014) Early selection of novel triploid hybrids of shrub willow with improved biomass yield relative to diploids. BMC Plant Biol. 14, 74.

Shukla, G.K. (1972) Some statistical aspects of partitioning genotype environmental components of variability. Heredity 29, 237-245.

Sims, J.T., Wolf, A.M. (1995) Recommended Soil Testing Procedures for the Northeastern United States. Agricultural Experiment Station Univ. of Delaware, Newark, Del., Northeastern Regional Pub. No. 493 (2nd edition).

Sixto, H., Gil, P., Ciria, P., Camps, F., Cañellas, I., Voltas, J. (2016) Interpreting genotype-by-environment interaction for biomass production in hybrid poplars under short-rotation coppice in Mediterranean environments. GCB Bioenergy, 8:1124-1135.

Sixto, H., Gil, P., Ciria, P., Camps, F., Sánchez, M., Cañellas, I., Voltas, J. (2014) Performance of hybrid poplar clones in short rotation coppice in Mediterranean environments: analysis of genotypic stability. GCB Bioenergy 6, 661-671.

Sleight, N.J., Volk, T.A., 2016. Recently bred willow (*Salix* spp.) biomass crops show stable yield trends over three rotations at two sites. Bioenerg. Res. 9:782-797.

Sleight, N.J., Volk, T.A., Johnson, G.A., Eisenbies, M.H., Shi, S., Fabio, E.S., Pooler, P.S. (2016) Change in yield between first and second rotations in willow (*Salix* spp.) biomass crops is strongly related to the level of first rotation yield. Bioenerg. Res. 9, 270-287.

Smart, L.B., Cameron, K.D. (2008) Genetic Improvement of Willow (*Salix* spp.) as a Dedicated Bioenergy Crop, in: Vermerris, W. (Ed.), Genetic Improvement of Bioenergy Crops. Springer New York, New York, NY, pp. 377-396.

Smart, L.B., Cameron, K.D. (2012) Shrub willow, in: Kole, C., Joshi, C.P., Shonnard, D.R. (Eds.), Handbook of Bioenergy Crop Plants. Taylor and Francis Group, Boca Raton, FL, pp. 687-708.

Smart, L.B., Cameron, K.D., Volk, T.A., Abrahamson, L.P. (2008) Breeding, selection, and testing of shrub willow as a dedicated energy crop, NABC Report 19 Agricultural Biofuels: Technology, Sustainability, and Profitability. National Agricultural Biotechnology Council, Ithaca, NY, pp. 85-92.

Smart, L.B., Volk, T.A., Lin, J., Kopp, R.F., Phillips, I.S., Cameron, K.D., White, E.H., Abrahamson, L.P. (2005) Genetic improvement of shrub willow (*Salix* spp.) crops for bioenergy and environmental applications in the United States. Unasylva 56, 51-55.

Smith, A.B., Cullis, B.R., Thompson, R. (2005) The analysis of crop cultivar breeding and evaluation trials: an overview of current mixed model approaches. J. Agric. Sci. 143, 449-462.

Stott, K.G. (1991) Nomenclature of the promising biomass coppice willows, *Salix* × *sericans* Tausch ex Kern., *Salix dasyclados* Wimm. and *Salix* 'Aquatica Gigantea'. Bot. J. Scotl. 46, 137-143.

U. S. Department of Energy (2011) U.S. billion-ton update: Biomass supply for a bioenergy and bioproducts industry. R.D. Perlack and B.J. Stokes (Leads). Oak Ridge National Laboratory. 227p., Oak Ridge, TN.

Volk, T.A., Abrahamson, L.P., Cameron, K.D., Castellano, P., Corbin, T., Fabio, E., Johnson, G., Kuzovkina-Eischen, Y., Labrecque, M., Miller, R., Sidders, D., Smart, L.B., Staver, K., Stanosz, G.R., Van Rees, K.C.J. (2011) Yields of willow biomass crops across a range of sites in North America. Asp. Appl. Biol. 112, 67-74.

Volk, T.A., Abrahamson, L.P., Nowak, C.A., Smart, L.B., Tharakan, P.J., White, E.H. (2006) The development of short-rotation willow in the northeastern United States for bioenergy and bioproducts, agroforestry and phytoremediation. Biomass Bioenerg. 30, 715-727.

Volk, T.A., Heavey, J.P., Eisenbies, M.H. (2016) Advances in shrub-willow crops for bioenergy, renewable products, and environmental benefits. Food Energy Secur. 97-106.

Walsh, M., de la Torre Ugarte, D., Shapouri, H., Slinsky, S. (2003) Bioenergy crop production in the United States: Potential quantities, land use changes, and economic impacts on the agricultural sector. Environ Resource Econ. 24, 313-333.

Wightman, J.L., Duxbury, J.M., Woodbury, P.B. (2015) Land quality and management practices strongly affect greenhouse gas emissions of bioenergy feedstocks. Bioenerg. Res. 8, 1681-1690.

CHAPTER 4

Contributions of environment and genotype to variation in shrub willow biomass composition

Indus. Crops Prod. (2017) 108:149-161

4.1 Abstract

Yield improvement of woody bioenergy crops has been the major focus of breeding programs, but biomass quality is also important for conversion to biofuels. Using high-resolution thermogravimetric analysis, the composition of biomass samples from two shrub willow (*Salix* spp.) yield trial networks representing two distinct datasets were examined. Dataset 1 consisted of 12 yield trials containing 10 genotypes that mainly represented early cultivars from the US breeding program. Dataset 2 consisted of five trial locations containing 19 genotypes from later breeding efforts. Variation in ash and hemicellulose content were largely controlled by genetic factors, while cellulose and lignin content were heavily influenced by environmental effects. Mean biomass composition traits for dataset 1 were 2.1% ash, 42.4% cellulose, 18.7% hemicellulose and 25.5% lignin. For dataset 2 mean traits were 1.9% ash, 43.2% cellulose, 17.7% hemicellulose and 25.7% lignin. Yield was negatively correlated with lignin content and positively correlated with cellulose content at the level of environment. Elite triploid hybrid cultivars have the potential to produce more cellulose per unit area because of higher yields and/or greater cellulose content. These findings suggest that selection of genotypes for improved yield, as well as selection of growing environment, can improve feedstock quality for biofuel production.

4.2 Introduction

Concerns over rising atmospheric carbon dioxide levels and the detrimental impacts associated with anthropogenic climatic change, coupled with interests in energy independence

have led to national efforts to explore alternatives sources of transportation fuels as viable replacements for petroleum-based fuels (Mussatto et al., 2010). Currently, traditional agricultural crops represent the major source of liquid biofuels, so called first-generation biofuels. In the US, current biofuel production mandates are targeted at approximately 68 GL per year, with the vast majority of total volume (80%) derived from maize grain (Environmental Protection Agency, 2015). Lignocellulosic material, in the form of plant stems and leaves, represents an abundant source of reduced carbon available for the production of second-generation biofuels. First-generation biofuels are widely criticized for competing with food production (Graham-Rowe, 2011) and result in lower greenhouse gas emission reductions compared with second-generation lignocellulosic feedstocks (Cherubini and Strømman, 2011). Due to these concerns, first-generation biofuel production in the US is capped at the present-day production level of 55 GL, and statutes mandate that second-generation biofuel production will match those levels by 2022. According to the most recent US government assessments of national biomass production potential, dedicated energy crops can provide the majority of biomass resources needed for bioenergy production in the coming decades (U.S. Department of Energy, 2016). However, this will require enormous investments in the expansion of operations across the supply chain.

Perennial woody bioenergy crops, such as shrub willow (*Salix* spp.), have key aspects relating to sustainability, including favorable greenhouse gas balances (Djomo et al., 2015), high rates of soil carbon storage (Cunniff et al., 2015; Pacaldo et al., 2014), low nutrient demand (Aronsson et al., 2014) and water quality benefits (Kuzovkina and Quigley, 2005; Styles et al., 2016). Recent life cycle analyses have concluded that yield is an important determining factor relating to greenhouse gas balances, and that fertilization will likely have detrimental effects (Caputo et al., 2014; Krzyżaniak et al., 2016). These benefits together with the pressing needs to

meet federal mandates and to control the concentration of carbon dioxide in the atmosphere will necessitate the expansion of robust, commercial-scale conversion technologies, which will demand steady supplies of feedstocks with uniform quality. Therefore, characterization of feedstock quality and an understanding of the sources of variability are of utmost importance.

Plant cell walls represent a vast reservoir of reduced carbon in the form of biopolymers, mostly cellulose, hemicellulose and lignin bound together in a complex network. Cellulose is the most abundant biopolymer on Earth and provides structural rigidity to plant cell walls. It is also a significant source of carbohydrates available for enzymatic hydrolysis and fermentation into liquid fuels, and represents the majority of substrates intended for second-generation biofuel production (Somerville et al., 2010). Hemicellulose is a heterogeneous biopolymer that adds strength to cells walls by linking cellulose microfibrils. Its composition differs greatly by plant species, but is mainly comprised of five-carbon sugar monomers and can therefore be a source of fermentable substrate using specialized or engineered microorganisms following chemical hydrolysis. Lignin has many important physiological roles in plants, including providing a hydrophobic surface in vascular tissues for water transport, and structural stability and resistance to disease and pest attack. However, it also presents a significant impediment to enzymatic cell wall depolymerization in liquid fuel production. Lignin has a higher energy density compared with cellulose and therefore is viewed as a desirable component for feedstocks used for thermal conversion. In contrast, ash, or mineral components of biomass, can be problematic for thermal and thermochemical conversion technologies, creating corrosion, slagging or fouling deposits. In biochemical conversion processes, ash can decrease pretreatment efficacy.

Woody biomass crops like poplar (*Populus* spp.) and willow have greater lignin and lower cellulose, hemicellulose and ash contents than perennial grasses (Karp and Shield, 2008)

and have typically been considered better feedstocks for thermal conversion. However, recent studies have demonstrated that genetic variability in biomass composition can affect potential biofuel production among species of poplar (Studer et al., 2011) and willow (Brereton et al., 2010; Serapiglia et al., 2013b), encouraging interest in exploring the genetic resources of these woody crops available for liquid fuel production. There is also great interest in the potential for genetic modification of cell wall composition in woody crops to improve conversion efficiency, however, in willow, unlike its close relative poplar, implementation of genetic modification for trait modification has not yet been reliably demonstrated (Stanton et al., 2014). Therefore, selection through traditional breeding techniques remains the most tractable mode for biomass quality improvement. Trait mapping with the aid of genomic resources for willow and poplar have recently been employed (Berlin et al., 2014; Brereton et al., 2010; Hanley et al., 2011), paving the way for marker-assisted selection.

Breeding efforts in North America have focused on improving yields by capturing heterosis in inter-specific hybrid crosses (Fabio et al., 2017b; Serapiglia et al., 2014), but with extensive genetic resources available, efforts have also been made to characterize variation in biomass composition among genotypes. Tharakan et al. (2003; 2005) found significant differences in wood density, bark concentration and ash content among a number of willow genotypes representing multiple species, many of which comprised the foundational material for the US breeding program (Smart et al., 2005). Serapiglia et al. (2013a) examined variability in biomass composition among 18 genotypes from initial breeding work at two locations in NY and found strong genotypic differences in cellulose, hemicellulose, lignin and ash content, as well as significant genotype-by-environment interactions (GEI) for some traits. Some important factors under genetic control that may affect biomass composition include stem diameter, bark

proportion (Adler et al., 2005) and biomass growth rate (Novaes et al., 2010). Besides genetic control, other factors affecting biomass quality include: stand age or rotation length (Adler et al., 2005; Stolarski et al., 2011), soil type (Krzyżaniak et al., 2015; Liu et al., 2016), fertilization rate (Larsen et al., 2016), and mechanical stress resulting in tension wood formation (Brereton et al., 2012). Despite a growing body of literature, most investigations have been conducted at single locations which prevents the evaluation of environmental and GEI effects.

The aim of this research was to quantify and describe the contributions of genotypic, environmental and GEI on shrub willow biomass composition variability, assessed by a high-throughput method. Biomass samples from two complementary, but unique yield trial networks that differ in their relative levels of geographic and genetic diversity, and represent different phases of breeding work in the US (Fabio et al., 2017a). The objectives were to 1) quantify the relative contributions of genetic, environmental and interaction factors in explaining variability in biomass traits; 2) explore patterns of correlation among the measured biomass traits; and 3) determine the degree to which biomass traits are influenced by genetic diversity and breeding.

4.3 Materials and methods

4.3.1 Yield trial networks and field sampling

The first yield trial network represented a combination of natural accessions and intra- and interspecific hybrids selected from early breeding efforts conducted at the State University of New York College of Environmental Science and Forestry, resulting from crosses made in 1998 and 1999. After selections were made based on initial field testing (Smart et al., 2008), yield trials were established in the US and Canada between 2005 and 2011, each containing between 16 and 30 genotypes planted in 78-plant plots. Each plot had three-double rows. Genotypes were laid out in a randomized complete block design with four replicates. The first-

year's growth was cut back and subsequently the trials were managed on a three-year rotation. Harvests were conducted on 18-22 plants per plot from the middle double row, either by manually cutting and weighing stems on hanging scales or by mechanical single-pass cut and chip harvesting and weighing chips in a bin fitted with load cells. A subsample of fresh biomass was collected from each plot, either as whole stems or chips depending on harvest method, and used to determine moisture content after oven drying at 65^{o}C to a constant weight. This was used in order to express yield on a dry-matter basis.

Because not all genotypes were present in all locations, and because biomass samples for compositional analysis (described below) were not available for all trials in this network, a reduced set of observations was selected that maximized geographic and genetic variability, while keeping the amount of missing data to a minimum (3.3% missing genotype-environment combinations). The final set of observations included 12 environments from the upper Midwestern and Northeastern US (Table 4.1) and represented 10 genotypes from five distinct pedigrees, or diversity groups, including two pure species and three inter-specific hybrid diversity groups (Table 4.2). Hereafter, measurements obtained from this first yield trial network will be referred to as dataset 1.

The second yield trial network consisted of five trials established in 2012 and 2013, each containing 24 to 27 genotypes representing a combination of commercial cultivars and high-yielding genotypes selected in a trial established in 2008 at Cornell University's New York State Agricultural Experiment Station in Geneva, NY (Serapiglia et al., 2014). The five trials were located in Escanaba, MI, Fredonia, NY (two trials), Geneva, NY and Rock Springs, PA, and were laid out in a randomized complete block design with four replicates (Table 4.3).

Table 4.1 Yield trial locations and environmental characteristics for Dataset 1.

Location	Code	Host institution	Year planted	LAT (DD)	LONG (DD)	Elev (m)	Pptn (mm)	GDD (°C)	Tmax (°C)	Tmin (°C)
Albion, MI	Albi	Michigan St Univ	2011	42.19	-84.73	312	674	1313	21	2
Belleville, NY	Bell	SUNY ESF	2005	41.98	-78.56	145	602	1274	21	2
Constableville, NY	Cons	SUNY ESF	2006	43.56	-75.53	513	842	812	19	-1.7
Escanaba, MI	Esca	Michigan St Univ	2008	45.77	-87.20	222	537	1016	19	-0.1
Fredonia, NY	Fred	Cornell Univ	2008	42.44	-79.29	255	662	1477	21	4.9
Lake City, MI	LakeC	Michigan St Univ	2010	44.30	-85.21	375	628	1214	21	1.1
Middlebury, VT	Midd	Middlebury College	2007	44.01	-73.20	114	766	1419	23	2.0
Potsdam, NY	Pots	SUNY Potsdam	2009	44.67	-74.97	134	714	1318	21	2
Savoy, IL	Savo	Univ Illinois	2010	40.07	-88.20	224	618	2097	25	6
Skandia, MI	Skan	Michigan St Univ	2009	46.36	-87.24	287	581	870	18	0.1
Storrs, CT	Stor	Univ Connecticut	2009	41.80	-72.23	198	848	1487	21	5.4
Tully, NY	Tull	SUNY ESF	2005	42.79	-76.12	379	816	1155	20	2

LAT, Latitude (DD, decimal degrees); LONG, longitude (DD, decimal degrees); Elev, elevation; Pptn, growing season (April-Oct) precipitation Y; GDD, annual growing degree days (base 10 °C); Tmax, growing season mean maximum temperature (°C); Tmin, annual mean minimum temperature (°C). Climatic variables are four-year means across the first harvest rotation.

Table 4.1 Continued

Location	**Rad** (MJ m^{-1} day^{-1})	**SOM** (%)	**pH**	**soilP** (mg kg^{-1})	**soilK** (mg kg^{-1})
Albion, MI	3790	2.0	6.37	5.6	128.3
Belleville, NY	3957	3.3	6.49	7.8	109.4
Constableville, NY	3572	8.2	5.66	1.0	71.8
Escanaba, MI	4008	2.8	6.10	1.0	30.3
Fredonia, NY	3895	3.6	4.80	8.3	122.5
Lake City, MI	3736	1.9	6.58	1.1	42.9
Middlebury, VT	3548	6.8	6.70	27.3	150.4
Potsdam, NY	3470	6.8	5.65	1.5	48.8
Savoy, IL	3977	2.4	6.27	1.0	84.7
Skandia, MI	3865	3.6	6.47	1.0	41.5
Storrs, CT	3785	3.4	6.12	1.2	72.8
Tully, NY	3674	3.2	4.99	2.4	77.2

Rad, cumulative growing season solar radiation; SOM, soil organic matter; pH, soil pH; soilP, soil phosphorus; soilK, soil potassium.

Table 4.2 Shrub willow genotype descriptions for dataset 1.

Epithet	Species/Pedigree	Mother	Father	Diversity Group[a]	Sex	Ploidy[b]	No. of trials
'Allegany'	*S. koriyanagi* × *S. purpurea*	SH3	95058	6b	F	2X	9
'Fish Creek'	*S. purpurea*	94006	94001	6a	M	2X	10
'Millbrook'	*S. purpurea* × *S. miyabeana*	95026	SX64	9	F	3X	10
'Oneida'	*S. purpurea* × *S. miyabeana*	94006	SX67	9	M	3X	10
'Otisco'	*S. viminalis* × *S. miyabeana*	SV2	SX64	8	F	3X	9
'Tully Champion'	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X	9
'Canastota'	*S. miyabeana*	SX61	SX64	5	M	4X	9
'Sherburne'	*S. miyabeana*	SX61	SX67	5	F	4X	10
'SX61'	*S. miyabeana*			5	F	4X	10
'SX64'	*S. miyabeana*			5	M	4X	10

[a]Diversity group codes are according to Gouker et al. (2015)
[b]Ploidy level estimated by flow cytometry (Serapiglia et al. 2015)

Table 4.3 Yield trial locations and environmental characteristics for dataset 2.

Variable	Units	Escanaba, MI	Fredonia, NY Amended	Fredonia, NY Control	Geneva, NY	Rock Springs, PA
LAT	(DD)	45.77	42.44	42.44	42.88	40.70
LONG	(DD)	-87.20	-79.29	-79.29	-77.00	-77.96
Elev	(m)	219.1	255.9	255.9	167.0	403.7
Pptn	(mm)	515.2	658.9	658.9	621.2	744.3
GDD	($^{\circ}$C)	981.7	1431.3	1431.3	1466.6	1651.5
Tmax	($^{\circ}$C)	19.3	20.9	20.9	21.4	22.0
Tmin	($^{\circ}$C)	-0.2	4.1	4.1	4.1	5.6
Rad	(MJ m^{-1} day^{-1})	3723.8	3624.1	3624.1	3513.7	3484.4
SOM	(%)	2.5	4.1	3.8	2.3	4.7
pH		7.0	6.2	6.0	6.5	5.6
soilP	(mg kg^{-1})	11.8	1.1	1.0	1.0	1.0
soilK	(mg kg^{-1})	71.1	104.6	83.9	44.7	90.6
soilCa	(mg kg^{-1})	1344.4	1526.9	1193.5	1414.6	1163.4
soilAl	(mg kg^{-1})	9.8	55.3	48.9	21.2	44.4

LAT, Latitude (DD, decimal degrees); LONG, longitude (DD, decimal degrees); Elev, elevation; Pptn, growing season (April-Oct) precipitation; GDD, annual growing degree days (base 10 ^{o}C); Tmax, growing season mean maximum temperature; Tmin, annual mean minimum temperature; Rad, growing season solar radiation; SOM, soil organic matter ; pH, soil pH; soilP, soil phosphorus; soilK, soil potassium; soilCa, soil calcium; soilAl, soil aluminum. Climatic variables are three or four-year means across the first harvest rotation.

Table 4.4 Shrub willow genotype descriptions for dataset 2.

Clone ID/ Epithet	Species/Pedigree	Mother	Father	Diversity Group[a]	Sex	Ploidy[b]	No. of trials
94006	*S. purpurea*	Natural		6a	F	2X	5
'Fish Creek'	*S. purpurea*	94006	94001	6a	M	2X	5
01X-265-019	*S. koriyanagi* × *S. purpurea*	SH3	94001	6b	M	2X	5
'Dimitrios'[c]	(*S. schwerinii* × *S. viminalis*) × *S. aeygyptiaca*	Tora		NA[e]	M	3X	5
02X-326-010	*S. miyabeana* × (*S. schwerinii* ×. *S. viminalis*)	9970-021	Olof	10	M	3X	5
02X-326-015	*S. miyabeana* × (*S. schwerinii* ×. *S. viminalis*)	9970-021	Olof	10	M	3X	5
'Victor'	*S. purpurea* × *S. miyabeana*	00-01-088	SX67	9	F	3X	5
05X-291-050	*S. purpurea* × *S. miyabeana*	00-01-088	SX67	9	M	3X	5
'Millbrook'	*S. purpurea* × *S. miyabeana*	95026	SX64	9	F	3X	5
'Saratoga'	*S. purpurea* × *S. miyabeana*	95026	SX64	9	F	3X	5
'Tonawanda'	(*S. koriyanagi* × *S. purpurea*) × *S. miyabeana*	99239-015	SX67	9b	F	3X	5
'Geneva Giant'	(*S. koriyanagi* × *S. purpurea*) × *S. miyabeana*	99239-015	SX67	9b	F	3X	5
'Sheridan'	*S. viminalis* × (*S. viminalis* × *S. miyabeana*)	SV2	99207-019	8	F	3X	5
'Seneca Chief'	*S. viminalis* × *S. miyabeana*	SV7	9970-037	8	F	3X	5
'Fabius'	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X	5
'Otisco'	*S. viminalis* × *S. miyabeana*	SV2	SX64	8	F	3X	5
'Preble'	*S. viminalis* × *S. miyabeana*	SV2	9970-037	8	F	3X	5
'SX61'	*S. miyabeana*	Natural		5	F	4X	5
'India'[d]	*S. dasyclados*	?		NA	M	6X	5

[a]Diversity group codes are according to Gouker et al. (2015).
[b]Ploidy level estimated by flow cytometry (Serapiglia et al. 2015).
[c]SW Seed cultivar (http://www.swseed.com/Products/Salix/)
[d]See Ngantcha et al. (2010).
[e]NA, not assigned.

In Fredonia, NY, the two trials were established side-by-side, one receiving soil fertility amendments of 1800 kg ha^{-1} of lime and 84 kg N, P and K ha^{-1} before and after the establishment year, while the other served as an unamended control. Most trials were planted in a double-row configuration with 48-plant plots, except for the Geneva, NY trial which was planted in a single row configuration with 24 plants per plot. As in the first yield trial network, first-year growth was cut back in all trials. The Geneva, NY trial and the two trials in Fredonia, NY were harvested on a two-year rotation, while the remaining three trials were harvested on a three-year rotation. Harvests were conducted on 20 plants per plot from the middle double row mechanically with a single-pass cut and chip harvester and the chips were weighed in a bin fitted with load cells. Among the five trials in dataset 2, there were 19 common genotypes, four of which were also present in the first trial network, and represented nine distinct pedigrees (diversity groups), including three pure species and six inter-specific hybrids (Table 4.4). There were no missing genotype-environment combinations, but there were five missing observations in the amended Fredonia, NY trial due to mechanical problems at harvest. Hereafter, measurements obtained from the second yield trial network will be referred to as dataset 2.

4.3.2 Biomass composition analysis

Samples for biomass compositional analysis from dataset 1 consisted mostly of chipped whole stems either from representative border plants, or a subsample of the moisture content samples described above. For the trials in Albion and Skandia, MI, and Potsdam, NY, biomass samples were collected by clipping a 10-cm stem segment from near the balance point (centroid) of a representative stem in a border row prior to harvest. For the early trials established in 2005 and 2006, only the first three replicate blocks were collected. In subsequent trials, samples from all four blocks were sampled, but to maintain a consistent number of replicates in each trial, one

block of the four blocks was randomly eliminated from consideration within each trial. For all trials in dataset 2, biomass samples were collected just prior to harvest by selecting a representative stem from a border plant in each plot and clipping a 10-cm stem segment from near the centroid of the stem. Samples were put into plastic bags and kept frozen until processed for biomass composition analysis. For dataset 2, density was estimated as the ratio of dry weight to volume, with the latter obtained by water displacement of fresh stem segments. Oven-dried chip samples or stem segments were milled using either a Wiley mill or a Retsch SM 300 (Retsch, Haan, Germany) cutting mill, followed by fine milling on an IKA MF 10 (IKA, Wilmington, NC) knife mill to pass a 0.5 mm mesh sieve. Fine-milled 10-mg samples were analyzed for biomass composition using high-resolution thermogravimetric analysis (HR-TGA) (Serapiglia et al., 2009; Serapiglia et al., 2013a; Serapiglia et al., 2015). All biomass composition data is presented on a percent dry-matter basis.

4.3.3 Statistical analysis

The yield trial network datasets were analyzed separately. Univariate analysis of each biomass trait was performed in SAS Version 9.4 using PROC MIXED, using restricted maximum likelihood (REML) for estimation of variance-covariance parameters. Variance components and their standard errors were estimated for the main effects of genotype and environment, the GEI and replicate nested within environment in an all random effects model. Wald Z-test statistics were used to assess the significance of each effect. Broad-sense heritability estimates were calculated on both a plot basis and a genotype-mean basis following Holland et al. (2003).

Genotypic and phenotypic correlation coefficients were estimated using a multivariate REML procedure where variance and covariance estimates for all pairwise trait combinations

were estimated following Holland (2006) using PROC MIXED in SAS version 9.4 (SAS Institute Inc., 2013). This method is preferred for these datasets, because some missing data (<5% for both datasets) created an unbalanced design, which can be handled in PROC MIXED. In addition, the mixed model REML approach allows for more direct computation of variance-covariance parameters as opposed to a MANOVA approach.

Initially attempts were made to run all random effects multivariate models, but there were problems with convergence. Model convergence was accomplished by considering environments and replicates (blocks) nested within environments as fixed effects, as recommended by Holland (2006), which increases computational efficiency by reducing the number of variance-covariance parameters to be estimated. The genotypic correlation coefficient between two traits was calculated using the genotypic covariance and variance estimates in the following expression:

$$r_{Gij} = \frac{cov_{Gij}}{\sqrt{\sigma^2_{Gi}\sigma^2_{Gj}}} \quad (1)$$

where cov_{Gij} is the genotypic covariance between traits *i* and *j*, and σ^2_{Gi} and σ^2_{Gj} are the genotypic variances for traits *i* and *j*, respectively. Genotypic correlation coefficients and their standard errors by using were estimated the unstructured correlations variance-covariance structure (TYPE=UNR) in the RANDOM statement and the COVTEST option in the PROC MIXED statement as suggested by Piepho and Möhring (2011). Wald Z-tests were used to test for significant differences from zero of the correlation coefficients.

Environmental correlation coefficients were also estimated in much the same way as the genotypic correlations, where the environment main effect covariance and variance estimates for two traits were substituted in equation 1. As previously mentioned, convergence issues arose

when all model effects were considered random, so in order to estimate the environmental effect variances and covariances for two traits efficiently, the main effect of genotype was considered fixed, while the main effect of environment, GEI and block within environment were considered random. In some instances of pairwise multivariate analyses, the PARMS option was needed using starting values for variance-covariance parameters from previous univariate and multivariate analyses to aid convergence (Piepho and Möhring, 2011). Environmental correlations and their standard errors were estimated using the TYPE=UNR variance-covariance structure option. Pairwise GEI correlation coefficients were also calculated by inserting the interaction variances and covariance for two traits into equation 1.

4.3.4 Graphical analysis

Best linear unbiased predictors (BLUPs) for each biomass trait from the univariate REML variance component analyses were collected separately by model effect into three separate matrices for genotype, environment and interaction effects. These matrices were each subjected to principal components analysis (PCA) based on the correlation matrix, because of differences in measurement units and scale among the five biomass traits. Principal components analyses were carried out using the Multivariate Methods platform in JMP® PRO version 12 (SAS Institute Inc., 2013). Biplots depicting trait loadings and model effect scores were constructed using the first two principal components.

Parallel coordinate plots (Wegman, 1990) were constructed to further illustrate the relationships among genotypes and environment for the four composition traits. First, for each dataset means were calculated across diversity groups (Tables 4.2 and 4.4) within each environment. Mean values were then normalized for each trait using the following formula:

$$x_{norm} = \frac{x_i - x_{min}}{x_{max} - x_{min}} \quad (2)$$

where x_i is the *i*th diversity group mean within an environment, and x_{max} and x_{min} represent the maximum and minimum group means across all environments. Normalized biomass trait values were plotted by environment. It should be stressed that these plots represent relative and not absolute patterns in the data. To preserve continuity and allow for visual comparisons between the two datasets, density measurements from dataset 2 were excluded from PCA biplots and parallel coordinate plots.

4.4 Results

4.4.1 Dataset 1

The univariate variance components analyses revealed significant environmental, genotypic and interactions for most of the biomass traits (Table 4.5). The main effect of environment accounted for the greatest proportion of variance for all traits and was largest for yield (70.4%) and cellulose content (65.3%). Ash and hemicellulose content had the lowest amount of environmental variance and the greatest levels of genotypic variance. Genotypic variances also accounted for a greater proportion of total variance compared with the GEI, suggesting a greater level of inheritance for these two traits. The main effect of genotype was not significant at the $P = 0.05$ level for lignin content or yield, however, the GEI accounted for nearly four to five times more variance than genotypic effects for these two traits. For cellulose content the main effect of genotype and the GEI accounted for nearly equal amounts of total variance, while the genotypic effect was nearly twice that of the GEI for ash content. Broad-sense heritability estimates were greatest for ash content, followed by cellulose and hemicellulose content which were nearly equivalent. Heritability estimates were relatively low

for yield and lignin content.

Table 4.5 Univariate REML analysis variance components and broad-sense heritability estimates for five biomass traits measured in the first yield trial network, represented as dataset 1.

	Biomass Trait								
	Ash Content			Cellulose Content			Hemicellulose Content		
Source	**Var[1]**	**SE**	**Pct**	**Var**	**SE**	**Pct**	**Var**	**SE**	**Pct**
ENV	0.44*	(0.20)	46.3	6.48*	(2.88)	65.3	0.31*	(0.15)	39.3
GEN	0.18*	(0.09)	18.3	0.77*	(0.42)	7.7	0.10*	(0.05)	12.5
GEN×ENV	0.10***	(0.03)	10.3	0.83***	(0.20)	8.3	0.07**	(0.02)	9.4
Rep(ENV)	0.03*	(0.01)	2.6	0.39**	(0.16)	3.9	0.07**	(0.03)	8.4
ERROR	0.21***	(0.02)	22.4	1.46***	(0.14)	14.7	0.24***	(0.02)	30.4
TOTAL	0.96			9.92			0.80		
Heritability (mean basis)			0.93			0.88			0.89
Heritability (plot basis)			0.37			0.25			0.24

[1]Var, variance; SE, standard error; Pct, percent, ENV, environment; GEN, genotype. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.0001$.

Table 4.5 Continued

	Biomass Trait					
	Lignin Content			Yield		
Source	**Var**	**SE**	**Pct**	**Var**	**SE**	**Pct**
ENV	1.25*	(0.58)	60.0	11.7*	(5.18)	70.4
GEN	0.05	(0.04)	2.4	0.10	(0.12)	0.6
GEN×ENV	0.19***	(0.05)	9.3	0.53***	(0.27)	3.2
Rep(ENV)	0.19**	(0.07)	9.3	0.81**	(0.34)	4.9
ERROR	0.4***	(0.04)	19.0	3.49***	(0.34)	21.0
TOTAL	2.09			16.63		
Heritability (mean basis)			0.65			0.41
Heritability (plot basis)			0.08			0.02

[1]Var, variance; SE, standard error; Pct, percent, ENV, environment; GEN, genotype. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.0001$.

At the species and species-hybrid level there were patterns in the differences among the biomass traits (Table 6). Ash content ranged from 1.6 to 2.7%, with ‘Fish Creek’, a *S. purpurea* cultivar and ‘Tully Champion’, a triploid hybrid, having low mean ash content (1.6%), while four *S. miyabeana* cultivars from diversity group 5, had the greatest ash content ($\bar{x}$ = 2.6%). Two *S. purpurea* × *S. miyabeana* triploid hybrids, ‘Millbrook’ and ‘Oneida’, had intermediate ash content ($\bar{x}$ = 1.9%), as did the *S. koriyanagi* × *S. purpurea* hybrid, ‘Allegany’ (2.0%). Two *S. viminalis* × *S. miyabeana* triploid hybrid cultivars, ‘Tully Champion’ and ‘Otisco’, had low mean ash content and high cellulose content. Hemicellulose and lignin content had relatively low levels of variability at the genotypic level. ‘Fish Creek’ had the greatest hemicellulose content, while the *S. miyabeana* cultivars had the lowest mean lignin content. While the main effect of genotype was not significant for yield in this dataset, the two *S. viminalis* × *S. miyabeana* cultivars that had the greatest mean cellulose content also had the greatest yields among the 10 cultivars in the dataset.

There were also geographic patterns among the biomass traits, with higher latitude locations tending to have greater ash and lignin content and simultaneously lower cellulose content and yields (Table 4.6). Hemicellulose content varied little by location, but also tended to be lower in low-yielding trials.

Multivariate REML variance and covariance parameter estimates allowed us to further dissect the genotypic, environmental and GEI effects through the calculation of correlation coefficients for each pair of traits within treatment factors (Table 4.7). Genotypic correlation analysis revealed significant negative correlations between ash and both cellulose and lignin content. A significant negative correlation existed between cellulose and hemicellulose content.

Table 4.6 Mean values among 10 genotypes and 12 environments for five biomass traits measured in the first yield trial network, represented as dataset 1.

Genotype	Diversity group	Ash (%)	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	Yield (Mg ha^{-1} yr^{-1})
'Canastota'	5	2.67	41.77	18.82	24.97	7.17
'Sherburne'	5	2.53	41.82	18.65	25.12	6.14
'SX61'	5	2.73	41.32	18.56	25.26	6.97
'SX64'	5	2.32	42.27	18.66	25.39	7.15
'Otisco'	8	1.76	44.16	18.31	25.39	7.80
'Tully Champion'	8	1.62	43.99	18.25	25.42	7.39
'Millbrook'	9	2.09	42.19	18.53	25.86	7.15
'Oneida'	9	1.79	42.61	18.97	25.42	7.11
'Fish Creek'	6a	1.62	41.95	19.40	25.84	7.13
'Allegany'	6b	2.00	41.97	18.51	25.80	5.89
CV (%)		20.13	2.23	1.79	1.19	8.07

Environment	Latitude (ºN)	Ash (%)	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	Yield (Mg ha^{-1} yr^{-1})
Skandia, MI	46.36	3.25	37.02	18.29	27.98	2.73
Escanaba, MI	45.77	1.88	42.95	18.80	24.82	6.28
Potsdam, NY	44.67	2.95	40.20	17.66	25.85	2.10
Lake City, MI	44.30	2.85	38.04	18.11	27.60	2.28
Middlebury, VT	44.01	2.05	43.53	18.07	25.30	12.84
Belleville, NY	43.79	2.23	45.00	19.01	24.29	10.94
Constableville, NY	43.56	2.05	43.40	18.96	25.04	7.28
Tully, NY	42.79	1.79	43.51	19.12	24.49	9.81
Fredonia, NY	42.44	1.54	42.20	19.05	25.09	7.09
Albion, MI	42.19	1.64	44.18	19.20	24.98	5.10
Storrs, CT	41.80	0.83	45.15	19.59	24.71	9.73
Savoy, IL	40.07	2.50	42.80	18.11	25.63	6.49
CV (%)		31.82	6.13	3.17	4.58	50.62
Overall mean		**2.12**	**42.39**	**18.67**	**25.45**	**6.99**

Table 4.7 Genotypic, environmental and genotype-by-environment interaction correlation coefficients (and standard errors) based on multivariate REML variance and covariance estimates for dataset 1.

Genotypic effects

	Cellulose	Hemi-cellulose	Lignin	Yield
Ash	**-0.74*** **	-0.09	**-0.87** **	-0.59
	(0.17)	(0.37)	(0.24)	(0.47)
Cellulose		**-0.56***	0.41	0.75
		(0.28)	(0.44)	(0.43)
Hemicellulose			0.30	0.09
			(0.43)	(0.56)
Lignin				0.29
				(0.67)

Environmental effects

	Cellulose	Hemi-cellulose	Lignin	Yield
Ash	**-0.82*** **	**0.89*** **	**0.80*** **	**-0.62** **
	(0.11)	(0.09)	(0.13)	(0.2)
Cellulose		**0.70*** **	**-0.96*** **	**0.79*** **
		(0.18)	(0.03)	(0.12)
Hemicellulose			**-0.65** **	**0.50***
			(0.2)	(0.25)
Lignin				**-0.74*** **
				(0.15)

Genotype-by-environment interaction effects

	Cellulose	Hemi-cellulose	Lignin	Yield
Ash	**-0.76*** **	-0.06	0.31	-0.11
	(0.08)	(0.2)	(0.17)	(0.25)
Cellulose		-0.01	**-0.71*** **	0.06
		(0.2)	(0.09)	(0.24)
Hemicellulose			-0.25	-0.03
			(0.18)	(0.29)
Lignin				0.13
				(0.26)

Significant correlations are bolded. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.0001$.

There was also a large positive, but non-significant correlation between cellulose content and yield. In contrast, environmental correlations were all significant at the $P < 0.05$ level or lower (Table 4.7). A notable difference compared with genotypic correlations is that ash content was strongly positively correlated with lignin content, but negatively correlated with all other biomass traits. Also, there was a significant positive correlation between cellulose and hemicellulose content. Cellulose content was also positively correlated with yield. The strongest relationship among biomass traits was a negative correlation between cellulose and lignin content (r_{env} = -0.96). Lignin content was also negatively correlated with yield. Far fewer significant correlations existed for GEI effects (Table 4.7). Hemicellulose content tended to have the lowest correlation coefficients with other biomass traits. As with genotypic and environmental effects, the GEI correlation between ash and cellulose content was strongly negative. The only other significant correlation was a negative relationship between cellulose and lignin content, which coincided with environmental effects.

Biplots from the PCA were used to project the relationships among biomass traits onto a two-dimensional space separately for the main effects of environment and genotype, and their interaction. For the main effect of environment the first PCA axis explained nearly 78% of the variance, and the dominant trend was the negative relationship between cellulose content and lignin and ash content (Figure 4.1a). Yield and hemicellulose content were positively associated with cellulose content. The lowest yielding trial locations, Scandia, MI, Lake City, MI and Potsdam, NY, grouped with ash and lignin content, while the higher yielding environments grouped near cellulose content. The second PCA axis represented a relatively small percentage of the total variance (13%), but hemicellulose content had the greatest loading on that axis.

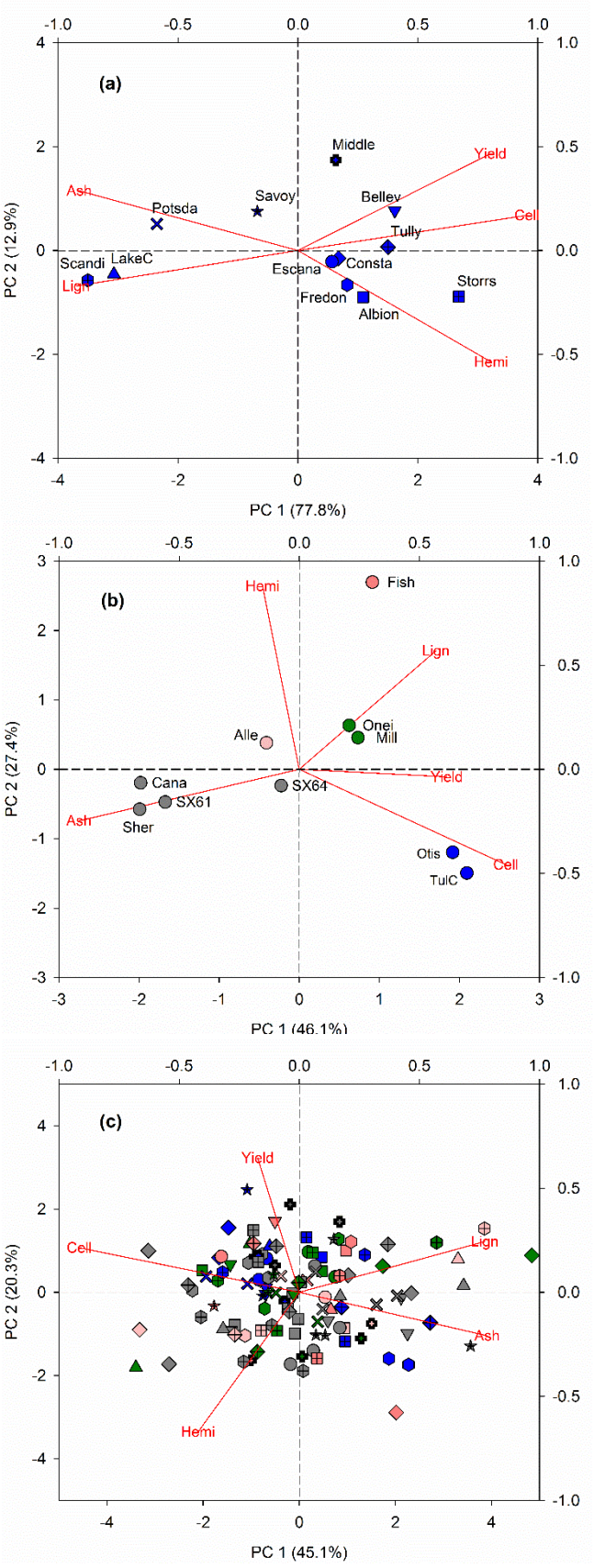


Figure 4.1 Principal component analysis biplots based on BLUP analysis for (a) main effect of environment, (b) main effect of genotype, and (c) the genotype-by-environment interaction for dataset 1. Top and right scales are for biomass trait loadings (in red, ash, cellulose, hemicellulose, or lignin content and yield). Bottom and left scales are for environment, genotype and interaction scores.

For the main effect of genotype, the first PCA axis explained a smaller proportion of total variance (46%) compared with the effect of environment, however it was also dominated by a negative relationship between ash and cellulose content (Figure 4.1b). The main difference in trait loading patterns between environmental and genotypic effects was the relationship between ash and lignin content. Environmental effects were stronger, and low yielding sites simultaneously exhibited higher ash and lignin. However, for genotypic effects the *S. miyabeana* cultivars likely contributed to the opposite pattern, since they had relatively low lignin content, but high ash content. Genotypic effects also displayed a positive association between yield and cellulose content. Hemicellulose content and to a lesser extent lignin content, dominated the pattern in the second PCA axis. Interestingly, genotype grouped rather well in PCA space, where most of the *S. miyabeana* cultivars grouped with ash and *S. viminalis* × *S. miyabeana* cultivars, 'Otisco' and 'Tully Champion' grouped with cellulose. The *S. purpurea* cultivar, 'Fish Creek', was characterized by having low ash content, but greater hemicellulose and lignin content. The *S. purpurea* × *S. miyabeana* cultivars co-located between the pure *S. miyabeana* and *S. purpurea* cultivars. The PCA analysis of the GEI accounted for a similar amount of total variance compared with genotypic effects (Figure 4.1c). The trait loadings largely reflected the patterns observed in that of the main effect of environment, except that the sign of the loadings along the first PCA axis were reversed. Hemicellulose content and yield showed strong, opposite loadings on the second PCA axis. There appeared to be fewer obvious patterns in the GEI effect scores.

Normalized biomass composition traits plotted by environment for the five diversity groups revealed patterns that were consistent by trial mean yield (Figure 4.2).

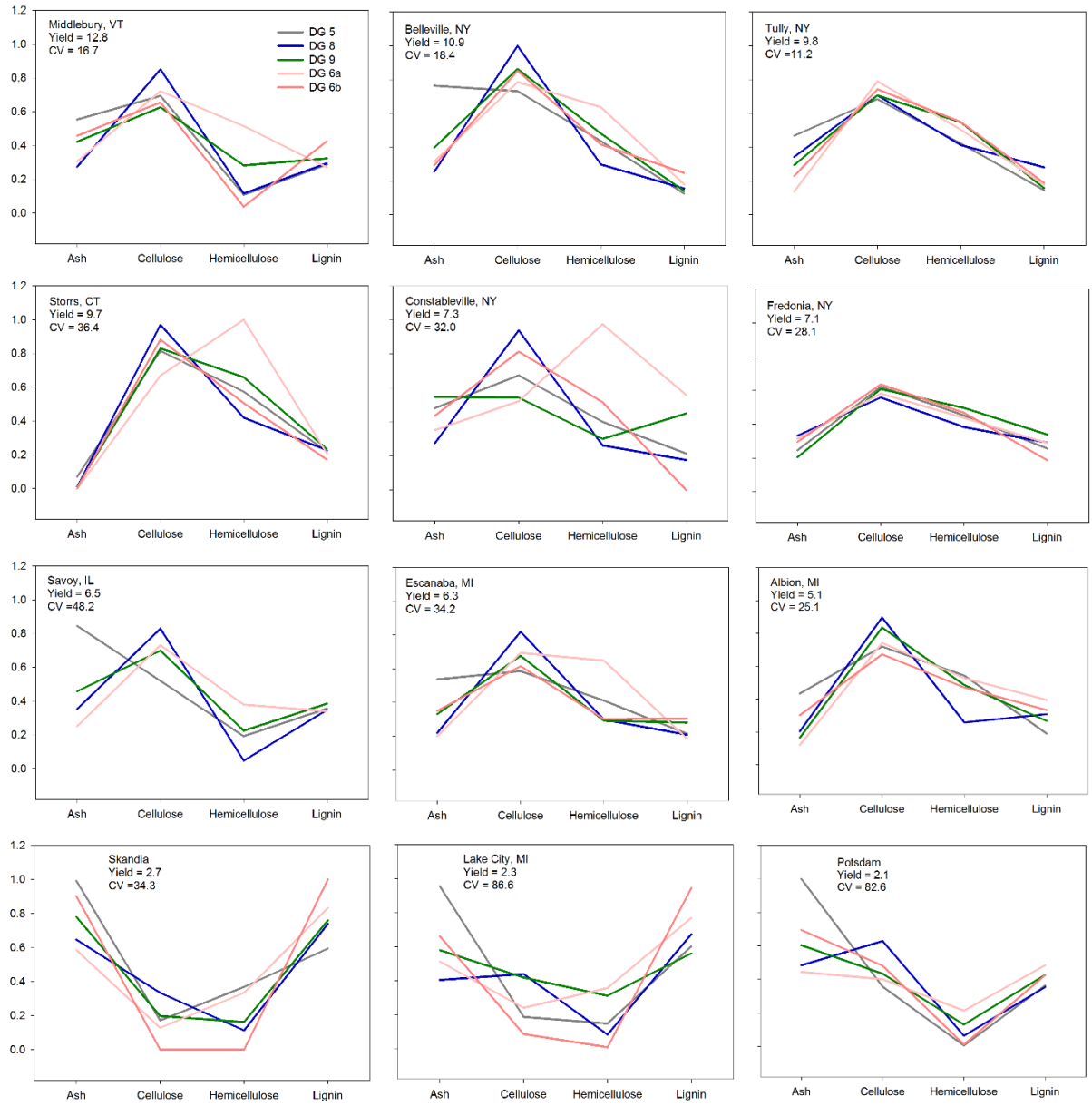


Figure 4.2 Parallel plots for each of the 12 environments in dataset 1. Lines represent normalized mean values calculated across all environments and summarized by diversity group (see Table 4.6) for four biomass composition traits (ash, cellulose, hemicellulose and lignin content). Trial mean yield and coefficient of variation (%) are given in the upper left for each environment.

Environments with higher mean yields tended to have peak cellulose content across diversity groups, except for diversity group 6a, representing the *S. purpurea* cultivar, 'Fish Creek', which had more prominent hemicellulose content values. In contrast, the three environments with mean yields below 3 Mg ha^{-1} yr^{-1} were characterized by greater normalized values of ash and lignin content. Diversity group 5, which represented the four *S. miyabeana* cultivars, consistently had the greatest normalized ash content in 10 of the 12 environments, while 'Fish Creek' and two *S. viminalis* × *S. miyabeana* cultivars (diversity group 8) often had the lowest ash content values. Diversity group 8 also tended to have the greatest cellulose content values in both high- and low-yielding environments.

4.4.2 Dataset 2

The main effect of genotype was significant for all six biomass traits in dataset 2 (Table 4.8). The main effect of environment was not significant according to Wald tests for any trait, due to relatively large standard errors and a small sample size. The main effect of environment accounted for over 50% of the total variance for cellulose content, lignin content and yield. In contrast, the main effect of genotype was the largest source of variance for ash and hemicellulose content. Density also had a relatively large proportion of genotypic variance, but environment was the largest source of variance. With the exception of density, the GEI was significant for all traits, but accounted for a small percentage of total variance. Broad-sense heritability estimates were greatest for hemicellulose and ash content, but all estimates were relatively high when computed on a genotype-mean basis.

The two *S. purpurea* genotypes in dataset 2, 'Fish Creek' and 94006, had ash content ≤1.35%, which were the lowest among the 19 genotypes in dataset 2, while the one *S. miyabeana* cultivar, 'SX61' again had the greatest ash content at 2.57% (Table 4.9).

Table 4.8 Univariate REML analysis variance components and broad-sense heritability estimates for five biomass traits measured in the second yield trial network, represented as dataset 2.

	Biomass Trait								
	Ash Content			Cellulose Content			Hemicellulose Content		
Source	**Var[1]**	**SE**	**Pct**	**Var**	**SE**	**Pct**	**Var**	**SE**	**Pct**
ENV	0.1	(0.08)	26.5	2.89	(2.11)	53.2	0.04	(0.04)	5.4
GEN	0.11**	(0.04)	29.7	0.52**	(0.22)	9.5	0.37**	(0.13)	47.9
GEN×ENV	0.03**	(0.01)	8.8	0.32**	(0.12)	5.9	0.06**	(0.02)	8.1
Rep(ENV)	0.01*	(0.01)	3.1	0.22*	(0.11)	4.1	0.03*	(0.02)	3.8
ERROR	0.12***	(0.01)	31.8	1.49***	(0.13)	27.3	0.27***	(0.02)	34.7
TOTAL	0.38			5.44			0.77		
Heritability (mean basis)			0.90			0.79			0.93
Heritability (plot basis)			0.42			0.22			0.53

[1]Var, variance; SE, standard error; Pct, percent, ENV, environment; GEN, genotype. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.0001$.

Table 4.8 Continued

	Biomass Trait								
	Lignin Content			Yield			Density		
Source	**Var**	**SE**	**Pct**	**Var**	**SE**	**Pct**	**Var (×10^4)**	**SE (×10^4)**	**Pct**
ENV	1.01	(0.73)	58.8	6.66	(4.88)	50.0	8.95	(6.54)	40.3
GEN	0.19**	(0.08)	11.1	1.49**	(0.64)	11.2	5.53**	(1.97)	24.9
GEN×E									
NV	0.07**	(0.03)	4.3	1.23**	(0.36)	9.2	0.25	(0.35)	1.1
Rep(ENV)	0.03	(0.02)	1.7	0.56*	(0.27)	4.2	0.79*	(0.42)	3.6
ERROR	0.42***	(0.04)	24.1	3.39***	(0.29)	25.5	6.68***	(0.58)	30.1
TOTAL	1.72			13.33			22.20		
Heritability (mean basis)			0.84			0.78			0.94
Heritability (plot basis)			0.28			0.24			0.44

[1]Var, variance; SE, standard error; Pct, percent, ENV, environment; GEN, genotype. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.0001$.

Table 4.9 Mean values among 19 genotypes and 5 environments for five biomass traits measured in the second yield trial network, represented as dataset 2.

Genotype	Diversity group	Ash (%)	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	Yield (Mg ha^{-1} yr^{-1})	Density (g cm^{-3})
'SX61'	5	2.57	42.02	18.19	24.89	6.92	0.51
94006	6a	1.35	43.04	18.08	25.74	5.16	0.44
'Fish Creek'	6a	1.27	43.23	18.42	25.65	6.36	0.45
01X-265-019	6b	1.69	44.37	17.06	25.99	5.97	0.44
'Fabius'	8	1.47	44.27	17.45	25.74	8.39	0.43
'Otisco'	8	1.74	44.76	17.10	25.94	7.16	0.45
'Preble'	8	2.03	42.46	18.30	25.37	8.63	0.48
'Seneca Chief'	8	2.44	41.81	18.14	25.57	7.46	0.45
'Sheridan'	8	2.19	42.58	17.71	26.31	6.16	0.45
05X-291-050	9	2.00	43.38	17.54	25.48	6.45	0.44
'Millbrook'	9	1.78	43.72	17.96	25.39	7.51	0.46
'Saratoga'	9	1.78	43.94	18.18	25.32	8.07	0.45
'Victor'	9	1.87	42.58	18.17	25.24	8.58	0.44
'Geneva Giant'	9b	2.15	42.21	17.34	26.07	7.56	0.51
'Tonawanda'	9b	1.94	43.09	17.76	25.29	8.53	0.43
02X-326-010	10	2.39	43.73	17.20	25.69	5.85	0.46
02X-326-015	10	2.18	43.16	18.17	25.82	6.32	0.47
'Dimitrios'	14	1.65	42.91	18.40	25.92	5.19	0.46
'India'	15	1.82	43.20	15.91	27.09	3.31	0.41
CV (%)		18.62	1.87	3.54	1.85	20.54	5.38

Environment	Latitude (ºN)	Ash (%)	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	Yield (Mg ha^{-1} yr^{-1})	Density (g cm^{-3})
Escanaba, MI	45.77	2.16	42.83	18.01	25.52	9.68	0.41
Geneva, NY	42.88	1.37	45.34	17.77	24.44	9.20	0.43
Fredonia, NY Amended	42.44	2.05	42.16	17.84	26.13	5.11	0.47
Fredonia, NY Unamended	42.44	2.14	41.09	17.36	27.17	3.55	0.48
Rock Springs, PA	40.70	1.83	44.50	17.73	25.30	6.42	0.48
CV (%)		17.18	4.00	1.33	3.95	38.71	6.72
Overall mean		**1.91**	**43.18**	**17.74**	**25.71**	**6.82**	**0.45**

The four *S. purpurea* × *S. miyabeana* genotypes had intermediate ash content levels ($\bar{x}$ = 1.87%).

The five *S. viminalis* × *S. miyabeana* had a wider range from a low of 1.47% ash for 'Fabius' to 2.44% for 'Seneca Chief". These triploid hybrids also varied widely in cellulose content, with 'Otisco' having the greatest (44.8%) and 'Seneca Chief' having the lowest (41.8%) mean cellulose content across all 19 genotypes. Lignin content had the least amount of variation of the five traits, but the lowest yielding cultivar, 'India', had the greatest lignin content. Consistent with dataset 1, the *S. miyabeana* cultivar had the lowest lignin content. The triploid hybrids had variable lignin content, but *S. purpurea* × *S. miyabeana* cultivars tended to have lower lignin content compared with *S. viminalis* × *S. miyabeana*. Tetraploid 'SX61' and triploid 'Geneva Giant' had the highest mean density values. With environment as the main effect, density increased with decreasing overall yield.

For dataset 2, there was a strong negative genotypic correlation between lignin content and yield (Table 4.10). Hemicellulose content was negatively correlated with lignin and cellulose content, but positively correlated with yield. Ash and cellulose content were also negatively correlated. Density was strongly and positively correlated with cellulose content and to a lesser extent lignin content. As in dataset 1, environmental correlation coefficients tended to be stronger compared with genotypic correlation coefficients in dataset 2. Cellulose content and yield were strongly, negatively correlated with lignin content, and cellulose and ash content were significantly, negatively correlated. Yield was positively correlated with hemicellulose and cellulose content. The GEI correlation coefficients also indicated negative relationships between cellulose content and ash and lignin content, but also a negative correlation between hemicellulose and lignin content.

Table 4.10 Genotypic, environmental and genotype-by-environment interaction correlation coefficients (and standard errors) based on multivariate REML variance and covariance estimates for dataset 2.

Genotypic effects

	Cellulose	Hemi-cellulose	Lignin	Yield	Density
Ash	**-0.50***	0.01	-0.27	0.20	-0.31
	(0.21)	(0.26)	(0.26)	(0.28)	(0.24)
Cellulose		**-0.46***	0.34	-0.13	**0.81*****
		(0.23)	(0.28)	(0.3)	(0.12)
Hemicellulose			**-0.76*****	**0.55****	**-0.46***
			(0.12)	(0.21)	(0.20)
Lignin				**-0.85*****	**0.46***
				(0.13)	(0.22)
Yield					-0.32
					(0.21)

Environmental effects

	Cellulose	Hemi-cellulose	Lignin	Yield	Density
Ash	**-0.88*****	-0.08	**0.82*****	-0.44	0.15
	(0.12)	(0.59)	(0.18)	(0.42)	(0.51)
Cellulose		0.47	**-0.97*****	**0.67***	-0.31
		(0.48)	(0.04)	(0.28)	(0.47)
Hemicellulose			-0.66	**0.94*****	**-0.87****
			(0.35)	(0.21)	(0.23)
Lignin				**-0.85*****	0.56
				(0.15)	(0.35)
Yield					**-0.92*****
					(0.09)

Genotype-by-environment interaction effects

	Cellulose	Hemi-cellulose	Lignin	Yield	Density
Ash	**-0.74*****	-0.17	0.41	-0.25	-0.40
	(0.11)	(0.23)	(0.22)	(0.2)	(0.55)
Cellulose		0.1	**-0.59****	0.3	0.47
		(0.26)	(0.18)	(0.22)	(0.58)
Hemicellulose			**-0.58****	-0.35	0.29
			(0.19)	(0.23)	(0.55)
Lignin				0.02	-0.18
				(0.25)	(0.57)
Yield					-0.47
					(0.47)

Significant correlations are bolded. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.0001$.

The PCA biplot for the main effect of environment in dataset 2 showed a similar pattern among trait loadings compared with dataset 1, with a strong negative relationship between cellulose content and ash and lignin content (Figure 4.3a). Yield was positively associated with cellulose content, while hemicellulose content had the strongest loading on the second PCA axis, followed by ash content. Geneva, NY was the second highest yielding environment and had a strong positive association with cellulose content. In contrast, the unamended trial at Fredonia, NY had the lowest overall yields and was strongly associated with lignin content. At the genotypic level, the triploid hybrids tended to cluster closer to yield on the first axis, while 'India', the lowest-yielding cultivar overall, was closely associated with lignin content (Figure 4.3b). The GEI effects PCA biplot showed very similar trait loadings as those in dataset 1 with the exception of a sign switch on the first axis. The GEI scores for *S. purpurea* × *S. miyabeana* genotypes tended to concentrate on the right side of the biplot, but otherwise there was little discernable pattern.

The greater amount of genetic diversity in dataset 2 seemed to introduce greater variability in the parallel coordinate plots compared with dataset 1 (Figure 4.4). The *S. dasyclados* cultivar, 'India', was characterized as having relatively high lignin content, low hemicellulose content, and extremely variable cellulose content. The Swedish cultivar, 'Dimitrios' ((*S. schwerinii* × *S. viminalis*) × *S. aeygyptiaca*) had high hemicellulose content values in high-yielding environments, greater than those of *S. purpurea* diversity group 6a, which had the greatest overall hemicellulose content in dataset 1. Triploid *S. viminalis* × *S. miyabeana* hybrids did not have the greatest cellulose content, as was the case in dataset 1.

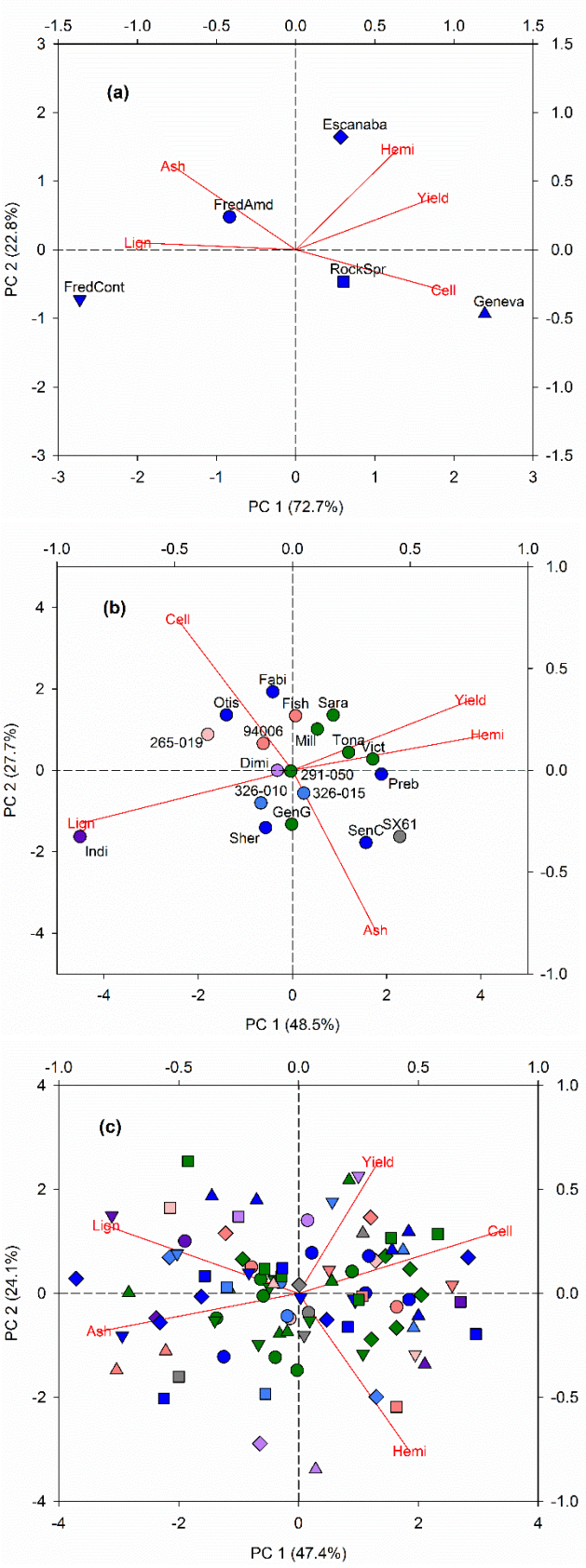


Figure 4.3 Principal component analysis biplots based on BLUP analysis for (a) main effect of environment, (b) main effect of genotype, and (c) the genotype-by-environment interaction for dataset 2. Top and right scales are for biomass trait loadings (in red, ash, cellulose, hemicellulose, or lignin content and yield). Bottom and left scales are for environment, genotype and interaction scores.

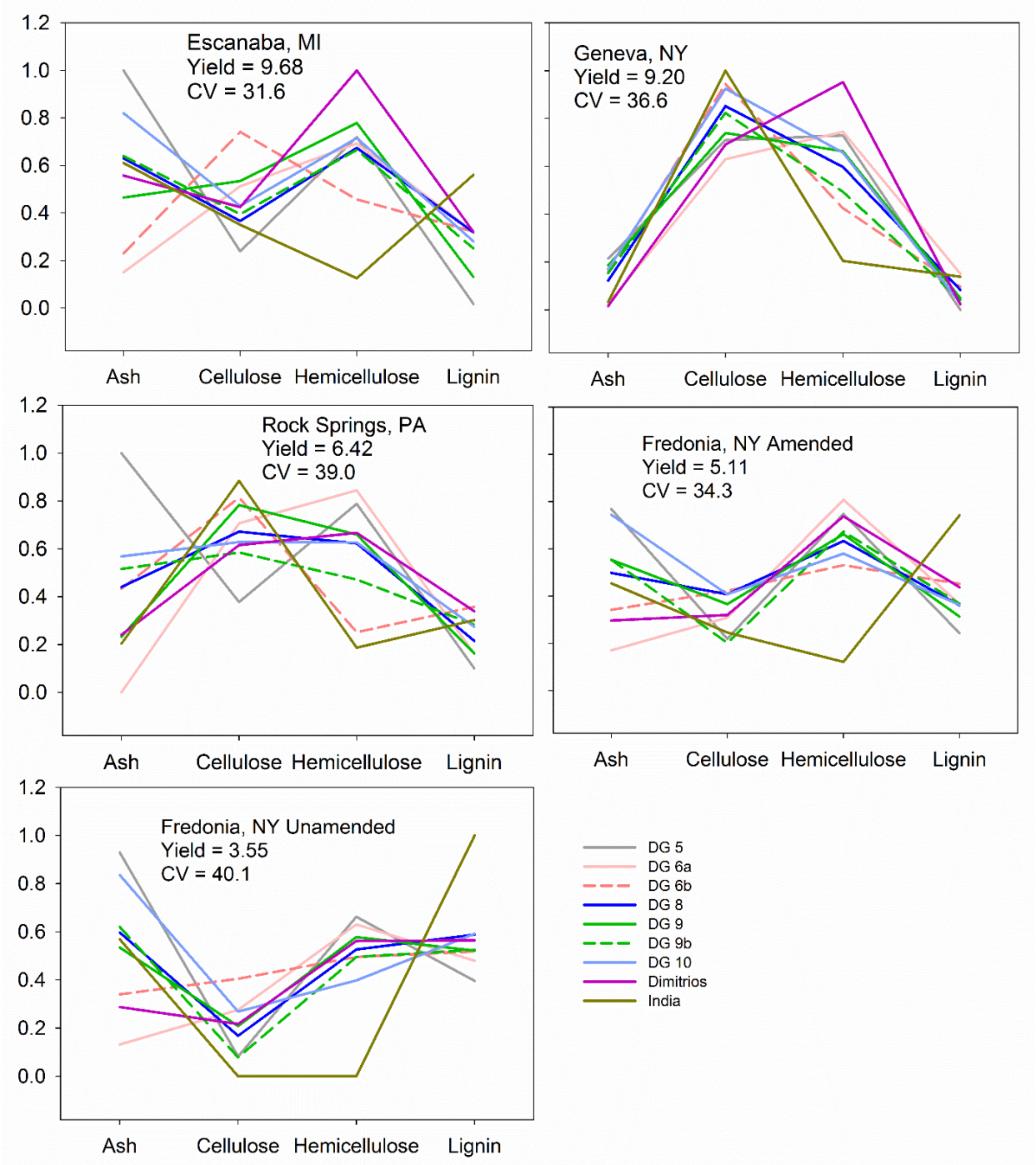


Figure 4.4 Parallel plots for each of the 5 environments in dataset 2. Lines represent normalized mean values calculated across all environments and summarized by diversity group (see Table 4.9) for four biomass composition traits (ash, cellulose, hemicellulose and lignin content). Trial mean yield and coefficient of variation (%) are given in the upper left for each environment.

However, there was a range in mean cellulose content among the five cultivars in this group, where ‘Otisco’ and ‘Fabius’ had the first and third greatest mean cellulose contents, respectively, out of all 19 cultivars in dataset 2. Also consistent with patterns in dataset 1, diversity group 5 (*S. miyabeana*) had the greatest ash content across all five environments in dataset 2. Geneva, NY, was ranked second in overall yield and the parallel plot had a similar convexed shape like the higher-yielding environments in dataset 1. The unamended Fredonia, NY trial was the lowest yielding environment and had a more concaved shape in the parallel plot, comparable to the lowest yielding environments in dataset 1.

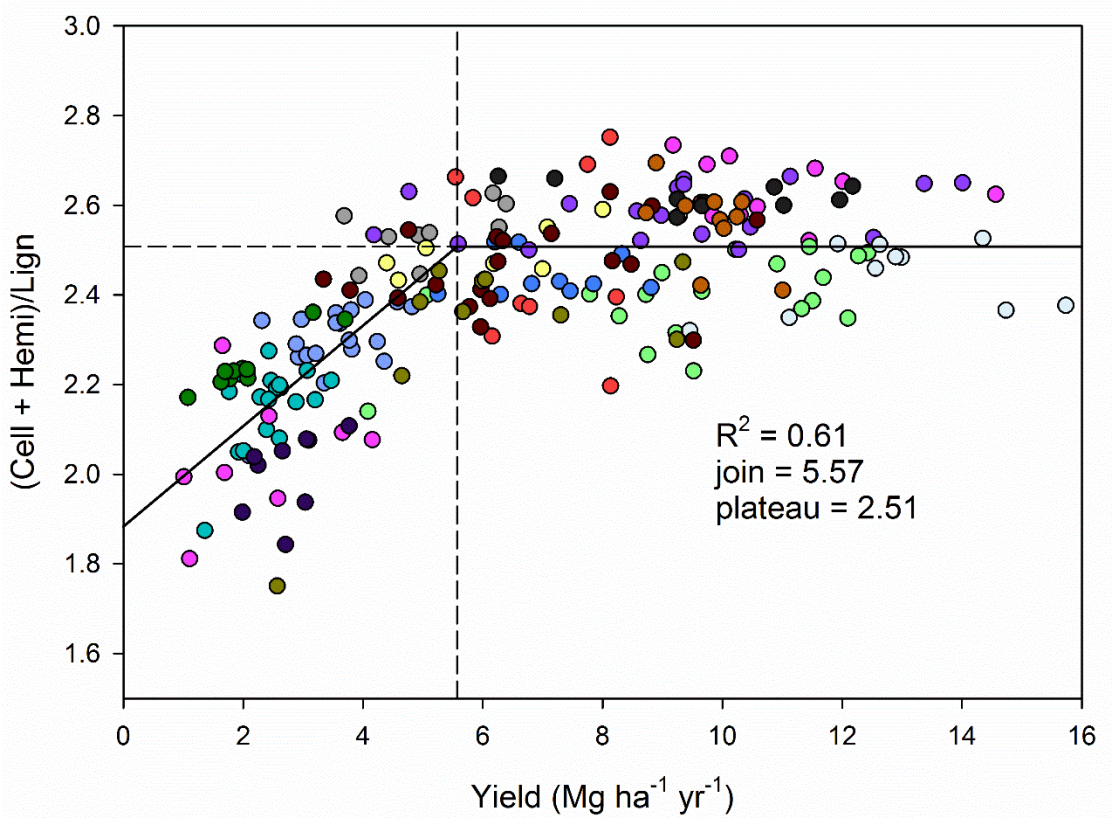


Figure 4.5 Linear-plateau regression analysis of the ratio of cellulose and hemicellulose combined over lignin content as a function of mean yield. Data points are genotype-by-environment means for datasets 1 and 2 combined, colored by environment. The join is the point on the x-axis where the two line segments meet, and the plateau is the corresponding point on the y-axis perpendicular to the join.

The ratio of the sum of structural carbohydrates (cellulose plus hemicellulose content) over lignin content was calculated, and plotted the ratio against yield for genotype-by-environment means using data from dataset 1 and 2 combined (Figure 4.5). This plot showed an initial steep incline followed by a clear leveling off in the ratio as yields increase. Therefore, a linear-plateau segmented regression was fit to the data with PROC NLIN using SAS software. This iterative process converged after 10 iterations with an R^2 of 0.61 and a joining point of the two line segments at a yield of 5.6 Mg ha^{-1} yr^{-1} (S.E. ± 0.28) and a plateau at 2.5 (SE ± 0.01) in the composition ratio. Above a yield of approximately 5.6 Mg ha^{-1} yr^{-1} the ratio stabilizes and the relative amounts of structural carbohydrates and lignin do not change with increasing yields.

4.5 Discussion

Strong evidence was found that in shrub willow, ash and hemicellulose content are under strong genetic control, while yield, cellulose and lignin content are largely influenced by environment. The broad environmental and genotypic variability present in these datasets allowed for the dissection of main effects and interactions. Three earlier studies reported seemingly contradictory results regarding the genetic variability in lignin, hemicellulose and cellulose concentration. Serapiglia et al. (2015) demonstrated significantly lower lignin content in tetraploid *S. miyabeana* and triploid hybrids compared with diploids in a genetic selection trial. Despite the large influence of environment, these findings tend to confirm this finding, especially for dataset 2. Zamora et al. (2014) analyzed biomass composition from a trial in Minnesota, US, containing eight willow cultivars, four of which were present in dataset 1, three being *S. miyabeana* cultivars. Those authors found a significant difference in lignin content, but not hemicellulose or cellulose content, as assayed by the NREL method. In contrast, Berthod et al. (2015) found no significant differences in lignin content by cultivar or environment in four

locations across Quebec, Canada, measured by ASTM and NREL methods. Three of the five cultivars used in that experiment were *S. miyabeana*. These examples point to the importance of testing diverse genetic material in multiple contrasting environments for evaluating biomass composition variation. While variance attributed to the GEI was statistically significant, it accounted for a low percentage of total variance among biomass traits, except for lignin content and yield in dataset 1. There is strong evidence of GEI for yield in willow (Fabio et al., 2017a; Fabio et al., 2017b), and in this study the ratio of GEI variance to genotypic variance was greatest for yield, followed by cellulose and lignin content. However the GEI PCA biplots revealed few discernible patterns, suggesting the interaction may contain a large level of noise relative to pattern.

Genotypic, environmental and GEI correlations exhibited largely similar patterns between the two datasets, but differences tended to align with the experimental diversity in those datasets. Dataset 1 with broad geographic diversity had the greatest number of significant environmental correlations. Dataset 2 had greater genetic diversity, and more significant genetic correlations were found, but there was still a greater number of significant environmental correlations, suggesting broad environmental influence on composition. One consistent result for both main effects and the GEI effect for both datasets was a strong negative correlation between ash and cellulose content. Higher yielding environments tended to have higher concentration of cellulose and lower concentration of ash, suggesting that greater biomass tended to dilute the ash content. The significant negative genotypic correlation between ash and lignin content for dataset 1 was likely due to species differences between *S. purpurea* and *S. miyabeana*, which are discussed in detail below. The dominant effect of environment otherwise likely drove the positive correlation between ash and lignin content, and their negative relationship with cellulose

content and yield, as evident in the PCA biplots and parallel plots. A similar pattern of negative relationships between ash and lignin content and cellulose content in PCA loadings was also reported by Krzyżaniak et al. (2015).

In an effort to explore specific environmental factors that may have influenced patterns in biomass composition, Pearson's correlation coefficients were calculated using environment mean composition values and the environmental variables reported in Tables 4.1 and 4.3. There was a significant negative correlation (r = -0.49, $P < 0.05$) between latitude and cellulose content, and a significant positive correlation between hemicellulose and solar radiation (r = 0.52, $P < 0.05$; Table 4.11). There were marginally significant ($P < 0.10$) negative correlations between longitude and ash and lignin content, and a marginally significant positive correlation between longitude and cellulose content. None of the climate and soil variables were found to have significant correlations with the composition traits, although eastern, lower latitudes were in general wetter and warmer (Tables 4.1 and 4.3). A recent analysis of yields from a portion of environments from dataset 1 showed that warmer and more humid environments were significantly positively correlated with yield and genotype-by-environment interactions, but also geography (Fabio et al., 2017b). This suggests that biomass quality and yields should improve in eastern, lower latitude locations, but more information is needed to determine the ideal growing range.

There is also strong evidence of a negative relationship between growth and lignin content for multiple of fast growing tree species that is under both genetic and environmental control (Novaes et al., 2010). Faster growing species of poplar inherently have been shown to have lower lignin content and nitrogen fertilization tends to enhance those differences (Li et al., 2012). In other poplar pot studies, fertilization decreased lignin content with an obviously

simultaneous increase in total biomass production (Novaes et al., 2009; Pitre et al., 2007).

Table 4.11 Pearson's correlation coefficients (and *P*-values) between composition traits and environmental variables (Tables 4.1 and 4.3) for datasets 1 and 2 combined (17 environments).

Variable	Ash	Cellulose	Hemi-cellulose	Lignin
LAT	*0.47*	**-0.49***	-0.04	0.29
	(0.05)	**(0.04)**	(0.88)	(0.25)
LONG	*-0.43*	*0.45*	0.14	*-0.44*
	(0.08)	*(0.06)*	(0.60)	*(0.08)*
Elev	0.03	-0.14	0.18	0.16
	(0.90)	(0.60)	(0.48)	(0.54)
Pptn	-0.39	0.31	0.33	-0.29
	(0.12)	(0.21)	(0.19)	(0.25)
GDD	-0.23	0.31	-0.24	-0.14
	(0.37)	(0.23)	(0.35)	(0.59)
Tmax	-0.10	0.27	-0.23	-0.12
	(0.69)	(0.30)	(0.36)	(0.65)
Tmin	-0.41	0.32	-0.14	-0.15
	(0.10)	(0.21)	(0.58)	(0.57)
Rad	0.04	-0.08	**0.52***	-0.03
	(0.87)	(0.77)	**(0.03)**	(0.89)
pH	0.24	-0.07	-0.28	0.24
	(0.35)	(0.78)	(0.28)	(0.34)
SOM	0.15	0.00	-0.12	-0.05
	(0.57)	(0.99)	(0.65)	(0.86)
soilP	-0.06	0.19	0.01	-0.19
	(0.81)	(0.46)	(0.96)	(0.46)
soilK	-0.31	0.40	0.18	-0.29
	(0.23)	(0.10)	(0.49)	(0.25)

LAT, Latitude (DD, decimal degrees); LONG, longitude (DD, decimal degrees); Elev, elevation; Pptn, growing season (April-Oct) precipitation; GDD, annual growing degree days (base 10 ^{o}C); Tmax, growing season mean maximum temperature; Tmin, annual mean minimum temperature; Rad, growing season solar radiation; SOM, soil organic matter ; pH, soil pH; soilP, soil phosphorus; soilK, soil potassium. * $P < 0.05$.

The soil fertility amendment treatment at the Fredonia, NY site in dataset 1 resulted in a 44% increase in overall yield, a 2.7% increase in cellulose content, and a 3.8% decrease in lignin content across all 19 genotypes. These changes in composition were much narrower than those

reported by Novaes et al. (2009), but the trend supports a notion that resource availability affects not only biomass production in woody species, but also partitioning patterns among cell wall constituents. It also implies that crops can be managed not only for improved growth potential, but also for biomass quality (Stolarski et al., 2015). Site quality differences in these datasets resulted in substantial differences in overall yield by location (2.1 to 12.8 Mg ha^{-1} yr^{-1} for dataset 1; 3.6 to 9.7 Mg ha^{-1} yr^{-1} for dataset 2), with the lowest yielding locations having the greatest lignin content. It has been demonstrated that juvenile willow stems generally have higher lignin content and lower cellulose and hemicellulose content compared with stems closer to harvest age (Serapiglia et al., 2015; Stolarski et al., 2011). Diminished growth rates due to poor growing conditions likely limited carbon available for wood formation, resulting in biomass composition more similar to young willow stems, with higher lignin and ash content. The relationship demonstrated in Figure 5 suggests that lignin production may have a base level needed for proper plant function, but when resources for photosynthesis are abundant, yields increase and a larger fraction of reduced carbon is allocated to cellulose biosynthesis, diluting lignin content. But at a yield level of approximately 5.6 Mg ha^{-1} yr^{-1}, the ratio of structural carbohydrates to lignin becomes relatively constant.

Hemicellulose variation was least affected by environment; on a genotypic level had high heritability estimates; and was negatively correlated with cellulose content at the genotypic level for both datasets. This suggests a high degree of species-specific partitioning patterns between these two major carbon sinks. In both datasets the *S. purpurea* diploid, ‘Fish Creek’, had the greatest overall mean hemicellulose content, although in dataset 2, ‘Dimitrios’ ((*S. schwerinii* × *S. viminalis*) × *S. aeygyptiaca*) had greater hemicellulose content in particular trial locations. At the genotypic level there was a lack of correlation of hemicellulose with other traits. While

cellulose is by far the dominant polysaccharide component of plant cell walls, the lack of genotypic correlation in these data suggests differential regulation of carbon allocation pathways where hemicellulose biosynthesis is reliant on availability of photosynthate, but that those biosynthesis pathways are regulated somewhat independently of cellulose pathways. Novaes et al. (2009) found that nitrogen supply not only increased cellulose concentration, but also hemicellulose, albeit at a significantly lower rate, in a hybrid poplar mapping population. It has been proposed that hemicellulose synthesis patterns are more similar to those of nonstructural carbohydrates (Schädel et al., 2010), which may help to explain the patterns observed in the PCA biplots, where hemicellulose loadings tended to be strongest in the second axis and were at near right angles to cellulose loadings, indicating a lack of correlation.

It has been speculated that greater ash content in woody biomass can be correlated with smaller diameter stems, resulting in a greater bark-to-wood ratio (Liu et al., 2016; Serapiglia et al., 2013a), as bark contains a greater concentration of ash constituents compared with wood (Klasnja et al., 2002; Tharakan et al., 2003). In an earlier study involving a large number of genotypes, Tharakan et al. (2005) reported that *S. purpurea* genotypes had on average approximately twice as many stems per plant compared with *S. miyabeana* genotypes (11.4 vs. 5.9 stem plant^{-1}, respectively; Table 4.12). The stems of *S. purpurea* genotypes had a mean diameter of 11.5 mm, while the *S. miyabeana* genotype stems were 15.4 mm in diameter. *S. purpurea* had a greater number of small stems and 15% lower biomass yield per plant, but only a slightly greater bark mass concentration (5.9 vs. 5.2%, respectively). However, in an analysis of ash content and elemental composition of biomass samples from the same trial (Tharakan et al., 2003), *S. purpurea* genotypes had 29% lower ash content in biomass samples compared with *S. miyabeana* (1.7 vs. 2.3%, respectively). This phenomenon was also observed in a separate study

using an expanded suite of genotypes, where *S. purpurea* genotypes had on average 15.1 stems plant^{-1} with a mean diameter of 12.6 mm, compared with 9.3 stems plant^{-1} and a mean diameter of 16.9 mm for *S. miyabeana* genotypes (Serapiglia et al., 2014). *Salix. purpurea* yields were 37% lower; however, *S. purpurea* mean ash content of bulk biomass was 1.5%, compared with 2.2% for *S. miyabeana* genotypes (Serapiglia et al., 2015). Furthermore, in the same trial *S. purpurea* × *S. miyabeana* hybrids showed intermediate trait values with 101 stems per plot and an ash content of 1.7%. Eich et al. (2015) examined one *S. purpurea* cultivar, 'Fish Creek' and one *S. miyabeana* cultivar, 'SX64', at two locations in New York and found no significant difference in stem bark concentrations between the two cultivars. These counterintuitive extremes in ash content relative to stem numbers between *S. purpurea* and *S. miyabeana* cultivars demonstrated in the present analysis and the above mentioned studies have been corroborated in commercial-scale harvest operations as well (Eisenbies et al., 2015). Perhaps the differences arise from higher concentrations of particular elements that contribute to ash in the bark of *S. miyabeana*. Tharakan et al. (2003) showed a very strong positive correlation between Ca and ash content, and concentrations of other elements differed only slightly between *S. miyabeana* and *S. purpurea.* Regardless, this evidence shows that for two of the prominent species used in willow breeding, there are strong genetic differences that are in contrast to the conventional view of the relationships between stem diameter/number and bark/ash content. This genetic control seems to have an additive pattern of inheritance, since species hybrids displayed intermediate phenotypes relative to their parents.

Table 4.12 Literature values comparing growth form and bark and ash concentrations for two prominent *Salix* species used in the US breeding program.

Parameter	Study	*S. miyabeana*	*S. purpurea*
Mean diam (mm)	Tharakan et al. 2005	15.4	11.5
	Serapiglia et al. 2014	16.9	12.6
No. stems plant^{-1}	Tharakan et al. 2005	5.9	11.4
	Serapiglia et al. 2014	9.2	15.2
Biomass (kg plant^{-1})	Tharakan et al. 2005	1.2	1.1
	Serapiglia et al. 2014	2.6	1.9
Bark conc. (%)	Tharakan et al. 2005	5.2	5.9
	Eich et al. 2015	12.0	12.6
Ash content (%)	Tharakan et al. 2003	2.34	1.67
	Eisenbies et al. 2014	2.40	1.35
	Serapiglia et al. 2015	2.15	1.47
	Current study	2.57	1.47

In dataset 1, the greatest yields were attained by triploid *S. viminalis* × *S. miyabeana* hybrids, 'Otisco' and 'Tully Champion'. These two top-yielding cultivars also had the greatest mean cellulose content. In dataset 2, triploid *S. viminalis* × *S. miyabeana*, and to a lesser extent, *S. purpurea* × *S. miyabeana* genotypes, had the greatest overall yields, but the relationship between cellulose and yield were more nuanced. For instance, 'Otisco' was also present in dataset 2 where it again had the greatest overall cellulose content, but was ranked ninth in yield among 19 genotypes. Cellulose content was multiplied by biomass yield to estimate a potential cellulose yield per unit of harvested area (Stolarski et al., 2015; Figure 4.6), 'Otisco' would yield 3.2 Mg ha^{-1} of cellulose based on dataset 2. 'Preble' and 'Victor', two other *S. viminalis* × *S. miyabeana* hybrids, had the top-two biomass yields, but relatively low cellulose content. However, when expressed as cellulose yield, 'Preble' and 'Victor' would produce 3.7 Mg ha^{-1} yr^{-}

^{1} of cellulose, approximately 14% more that ‘Otisco’ on average. Those two cultivars also had relatively low lignin content. Another triploid *S. viminalis* × *S. miyabeana*, ‘Fabius’, was ranked 4th in overall yield and 3rd in cellulose content, but would yield 3.7 Mg ha^{-1} yr^{-1} of cellulose as well. This implies that selection of genotypes based on yield performance can result in simultaneous selection for greater cellulose content and/or lower lignin content, which will presumably result in more efficient conversion to biofuels.

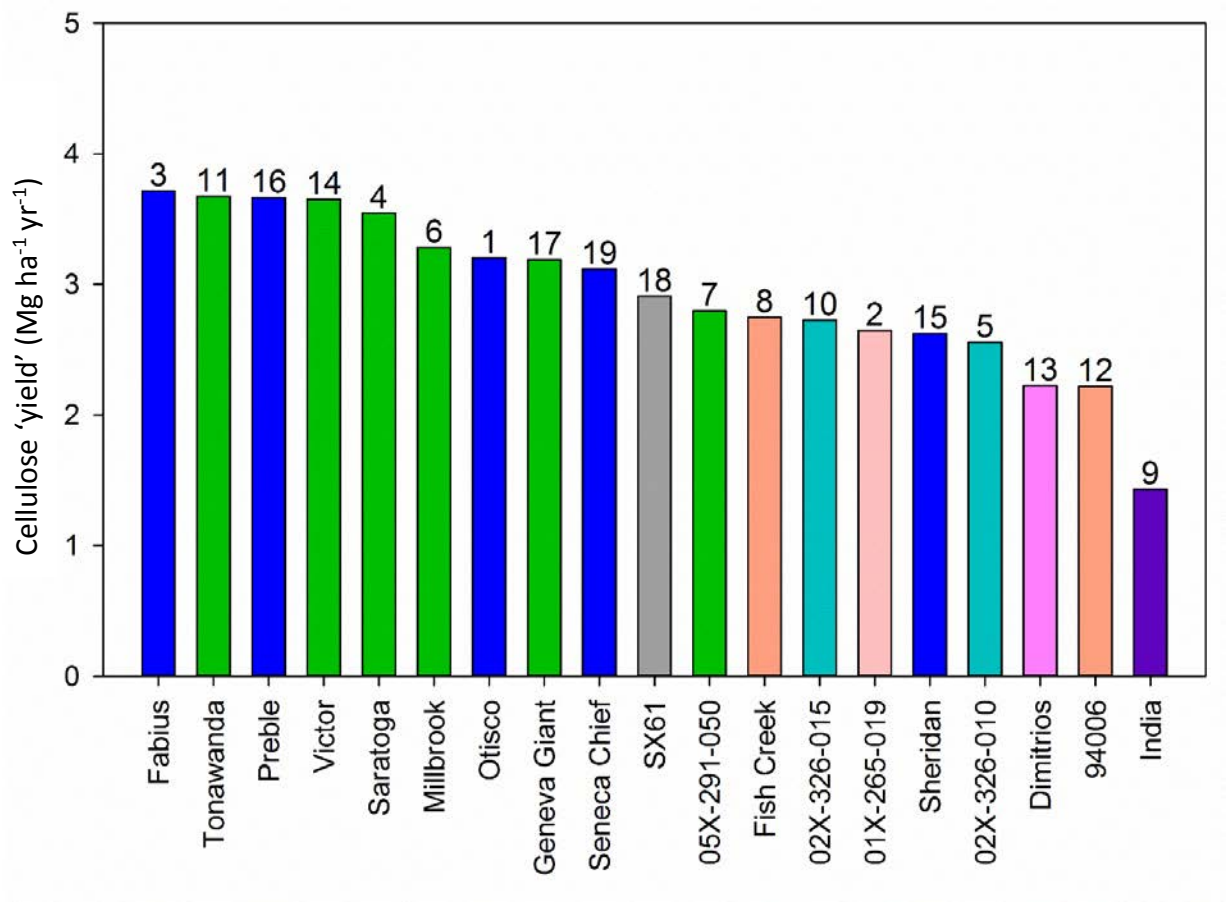


Figure 4.6 Cellulose ‘yield’ for dataset 2 calculated by multiplying cellulose concentration by the biomass yield for each cultivar. Data are means across five trials. Bars are color coded by diversity group (see Table 4.4). Numbers above bars represent each cultivar’s rank in cellulose content, 1 to 19, with 1 being the cultivar with the greatest mean cellulose content.

Information on conversion of willow biomass to liquid fuel is limited, but some recent studies have tested differences among cultivars. Serapiglia et al. (2013b) analyzed samples from

a number of cultivars present in the current study for biomass composition and enzymatic saccharification and found a strong correlation between cellulose content and sugar release. These authors noted that while 'Preble' ranked 22nd out of 30 genotypes for cellulose content, it had the second highest sugar yield per unit of biomass. Ray et al. (2012) analyzed a number of genotypes from the UK and Swedish breeding programs and found significant differences for enzymatic saccharification, but these were not related to lignin content. Clearly there is evidence that genetic resources available to shrub willow breeding programs can be exploited for selection and improvement of genotypes for biofuel production, and new molecular genetic analyses, such as QTL mapping, promise to accelerate this process (Brereton et al., 2010). However the relationships among biomass components and the underlying biochemical properties that affect the variation in biofuel yield potential remain important areas of research.

4.6 Conclusions

The current study underlines the impact of genotypic and environmental effects on variation in shrub willow biomass quality. The use of HR-TGA as a high-throughput method for composition analysis allowed us to leverage a large collection of biomass samples composed of diverse genotypes grown in a multitude of environments. Ash and hemicellulose content were found to be largely under genetic control, and differences in ash content among species did not support the conventional view for how plant form affects bark and ash content. Intermediate ash levels in interspecific hybrids suggests an additive pattern of inheritance for this trait. Numerous significant correlations were found among biomass composition traits, but the most dominant patterns were the negative relationships between cellulose and lignin content, which seemed to be largely under environmental control. Also, at the environmental level, cellulose content and yield were positively correlated, indicating that more productive stands should yield greater

quantities of cellulose. Triploid hybrids tended to have average to above average cellulose content and greater yields, resulting in greater potential cellulose yields per unit area. The genetic variation in wood composition as well as the ability to modify composition via management, indicate that both new genetic resources and management can be used to optimize biofuel conversion efficiency.

4.7 References

Adler A, Verwijst T, Aronsson P (2005) Estimation and relevance of bark proportion in a willow stand. Biomass Bioenerg. 29:102-113.

Aronsson P, Rosenqvist H, Dimitriou I (2014) Impact of Nitrogen Fertilization to Short-Rotation Willow Coppice Plantations Grown in Sweden on Yield and Economy. Bioenerg Res. 7:993-1001.

Berlin S, Ghelardini L, Bonosi L, Weih M, Ronnberg-Wastljung AC (2014) QTL mapping of biomass and nitrogen economy traits in willows (Salix spp.) grown under contrasting water and nutrient conditions. Mol Breed. 34:1987-2003.

Berthod N, Brereton NJB, Pitre FE, Labrecque M (2015) Five willow varieties cultivated across diverse field environments reveal stem density variation associated with high tension wood abundance. Front Plant Sci. 6:948.

Brereton NB, Pitre F, Hanley S, Ray M, Karp A, Murphy R (2010) QTL mapping of enzymatic saccharification in short rotation coppice willow and its independence from biomass yield. Bioenerg Res. 3:251-261.

Brereton NJ, Ray MJ, Shield I, Martin P, Karp A, Murphy RJ (2012) Reaction wood – a key cause of variation in cell wall recalcitrance in willow. Biotechnol Biofuels. 5:83.

Buchholz T, Volk T (2013) Profitability of willow biomass crops affected by incentive programs. Bioenerg Res. 6:53-64.

Caputo, J., Balogh, S.B., Volk, T.A., Johnson, L., Puettmann, M., Lippke, B., O'Neil, E. (2014) Incorporating uncertainty into a life cycle assessment (LCA) model of short-rotation willow biomass (*Salix* spp.) crops. Bioenerg Res. 7:48-59.

Cherubini F, Strømman AH (2011) Life cycle assessment of bioenergy systems: State of the art and future challenges. Bioresour Technol. 102:437-451.

Cunniff J, Purdy SJ, Barraclough TJP, Castle M, Maddison AL, Jones LE, Shield IF, Gregory AS, Karp A (2015) High yielding biomass genotypes of willow (Salix spp.) show differences in below ground biomass allocation. Biomass Bioenerg. 80:114-127.

Djomo SN, Witters N, Van Dael M, Gabrielle B, Ceulemans R (2015) Impact of feedstock, land use change, and soil organic carbon on energy and greenhouse gas performance of biomass cogeneration technologies. Appl Energy. 154:122-130.

Eich S, Volk TA, Eisenbies MH (2015) Bark content of two shrub willow cultivars grown at two sites and relationships with centroid bark content and stem diameter. Bioenerg Res. 8:1661-1670.

Eisenbies MH, Volk TA, Posselius J, Shi S, Patel A (2015) Quality and variability of commercial-scale short rotation willow biomass harvested using a single-pass cut-and-chip forage harvester. Bioenerg Res. 8:546-559.

Environmental Protection Agency (2015) Renewable Fuel Standard Program: Standards for 2014, 2015, and 2016 and Biomass-Based Diesel Volume for 2017. 80 CFR part 40

Fabio ES, Kemanian AR, Montes F, Miller RO, Smart LB (2017a) A mixed model approach for evaluating yield improvements in interspecific hybrids of shrub willow, a dedicated

bioenergy crop. Ind Crops Prod. 96:57-70.

Fabio ES, Volk TA, Miller RO, Serapiglia MJ, Gauch HG, Van Rees KCJ, Hangs RD, Amichev BY, Kuzovkina YA, Labrecque M, Johnson GA, Ewy RG, Kling GJ, Smart LB (2017b) Genotype × environment interaction analysis of North American shrub willow yield trials confirms superior performance of triploid hybrids. GCB Bioenergy. 9:445-459.

Graham-Rowe D (2011) Agriculture: Beyond food versus fuel. Nature. 474:S6-S8.

Hanley SJ, Pei MH, Powers SJ, Ruiz C, Mallott MD, Barker JHA, Karp A (2011) Genetic mapping of rust resistance loci in biomass willow. Tree Genet Genomes. 7:597-608.

Holland JB (2006) Estimating genotypic correlations and their standard errors using multivariate restricted maximum likelihood estimation with SAS Proc MIXED. Crop Sci. 46:642-654.

Holland JB, Nyquist WE, Cervantes-Martínez CT (2003) Estimating and interpreting heritability for plant breeding: an update. Plant Breed Rev. 22:9-112.

Karp A, Shield I (2008) Bioenergy from plants and the sustainable yield challenge. New Phytol. 179:15-32.

Klasnja B, Kopitovic S, Orlovic S (2002) Wood and bark of some poplar and willow clones as fuelwood. Biomass Bioenerg. 23:427-432.

Krzyżaniak, M., Stolarski, M.J., Szczukowski, S., Tworkowski, J. (2016) Life cycle assessment of new willow cultivars grown as feedstock for integrated biorefineries. Bioenerg Res. 9:224-238.

Krzyżaniak M, Stolarski MJ, Szczukowski S, Tworkowski J, Bieniek A, Mleczek M (2015) Willow biomass obtained from different soils as a feedstock for energy. Ind Crops Prod. 75, Part B:114-121.

Kuzovkina YA, Quigley MF (2005) Willows beyond wetlands: Uses of Salix L. species for

environmental projects. Water Air Soil Pollut. 162:183-204.

Larsen SU, Jørgensen U, Kjeldsen JB, Lærke PE (2016) Effect of fertilisation on biomass yield, ash and element uptake in SRC willow. Biomass Bioenerg. 86:120-128.

Li H, Li M, Luo J, Cao X, Qu L, Gai Y, Jiang X, Liu T, Bai H, Janz D, Polle A, Peng C, Luo Z-B (2012) N-fertilization has different effects on the growth, carbon and nitrogen physiology, and wood properties of slow- and fast-growing *Populus* species. J Exp Bot. 63:6173-6185.

Liu N, Jørgensen U, Lærke PE (2016) Concentrations of chemical elements in willow biomass depend on clone, site and management in the field. Bioenerg Res. 9:1216-1230.

Mussatto SI, Dragone G, Guimarães PMR, Silva JPA, Carneiro LM, Roberto IC, Vicente A, Domingues L, Teixeira JA (2010) Technological trends, global market, and challenges of bio-ethanol production. Biotechnol Adv. 28:817-830.

Novaes E, Kirst M, Chiang V, Winter-Sederoff H, Sederoff R (2010) Lignin and biomass: A negative correlation for wood formation and lignin content in trees. Plant Physiol. 154:555-561.

Novaes E, Osorio L, Drost DR, Miles BL, Boaventura-Novaes CRD, Benedict C, Dervinis C, Yu Q, Sykes R, Davis M, Martin TA, Peter GF, Kirst M (2009) Quantitative genetic analysis of biomass and wood chemistry of Populus under different nitrogen levels. New Phytol. 182:878-890.

Pacaldo R, Volk T, Briggs R (2014) Carbon sequestration in fine roots and foliage biomass offsets soil CO2 effluxes along a 19-year chronosequence of shrub willow (Salix x dasyclados) biomass crops. Bioenerg Res. 7:769-776.

Piepho H-P, Möhring J (2011) On estimation of genotypic correlations and their standard errors

by multivariate REML using the MIXED procedure of the SAS system. Crop Sci. 51:2449-2454.

Pitre FE, Pollet B, Lafarguette F, Cooke JEK, MacKay JJ, Lapierre C (2007) Effects of increased nitrogen supply on the lignification of poplar wood. J Agric Food Chem. 55:10306-10314.

Ray MJ, Brereton NJB, Shield I, Karp A, Murphy RJ (2012) Variation in cell wall composition and accessibility in relation to biofuel potential of short rotation coppice willows. Bioenerg Res. 5:685-698.

SAS Institute Inc. 2013. SAS Institute, Inc, Cary, NC, USA.

Schädel C, Richter A, Blöchl A, Hoch G (2010) Hemicellulose concentration and composition in plant cell walls under extreme carbon source–sink imbalances. Physiol Plant. 139:241-255.

Serapiglia M, Cameron K, Stipanovic A, Smart L (2009) Analysis of biomass composition using high-resolution thermogravimetric analysis and percent bark content for the selection of shrub willow bioenergy crop varieties. Bioenerg Res. 2:1-9.

Serapiglia MJ, Cameron KD, Stipanovic AJ, Abrahamson LP, Volk TA, Smart LB (2013a) Yield and woody biomass traits of novel shrub willow hybrids at two contrasting sites. Bioenerg Res. 6:533-546.

Serapiglia MJ, Gouker FE, Hart JF, Unda F, Mansfield SD, Stipanovic AJ, Smart LB (2015) Ploidy level affects important biomass traits of novel shrub willow (Salix) hybrids. Bioenerg Res. 8:259-269.

Serapiglia MJ, Gouker FE, Smart LB (2014) Early selection of novel triploid hybrids of shrub willow with improved biomass yield relative to diploids. BMC Plant Biol. 14:74.

Serapiglia MJ, Humiston MC, Xu H, Hogsett DA, Mira de Orduña R, Stipanovic AJ, Smart LB (2013b) Enzymatic saccharification of shrub willow genotypes with differing biomass composition for biofuel production. Front Plant Sci. 4:57.

Smart LB, Cameron KD, Volk TA, Abrahamson LP. 2008. Breeding, selection, and testing of shrub willow as a dedicated energy crop. In NABC Report 19 Agricultural Biofuels: Technology, Sustainability, and Profitability. National Agricultural Biotechnology Council, Ithaca, NY, pp 85-92.

Smart LB, Volk TA, Lin J, Kopp RF, Phillips IS, Cameron KD, White EH, Abrahamson LP (2005) Genetic improvement of shrub willow (*Salix* spp.) crops for bioenergy and environmental applications in the United States. Unasylva. 56:51-55.

Somerville C, Youngs H, Taylor C, Davis SC, Long SP (2010) Feedstocks for lignocellulosic biofuels. Science. 329:790-792.

Stanton BJ, Serapiglia MJ, Smart LB (2014) The domestication and conservation of Populus and Salix genetic resources. Poplars and willows: trees for society and the environment Wallingford, UK: CAB International:124-199.

Stolarski MJ, Krzyżaniak M, Łuczyński M, Załuski D, Szczukowski S, Tworkowski J, Gołaszewski J (2015) Lignocellulosic biomass from short rotation woody crops as a feedstock for second-generation bioethanol production. Ind Crops Prod. 75, Part B:66-75.

Stolarski MJ, Szczukowski S, Tworkowski J, Wróblewska H, Krzyżaniak M (2011) Short rotation willow coppice biomass as an industrial and energy feedstock. Ind Crops Prod. 33:217-223.

Studer MH, DeMartini JD, Davis MF, Sykes RW, Davison B, Keller M, Tuskan GA, Wyman CE (2011) Lignin content in natural Populus variants affects sugar release. Proc Natl Acad

Sci USA. 108:6300-6305.

Styles D, Börjesson P, D'Hertefeldt T, Birkhofer K, Dauber J, Adams P, Patil S, Pagella T, Pettersson LB, Peck P, Vaneeckhaute C, Rosenqvist H (2016) Climate regulation, energy provisioning and water purification: Quantifying ecosystem service delivery of bioenergy willow grown on riparian buffer zones using life cycle assessment. Ambio. 45:872-884.

Tharakan PJ, Volk TA, Abrahamson LP, White EH (2003) Energy feedstock characteristics of willow and hybrid poplar clones at harvest age. Biomass Bioenerg. 25:571-580.

Tharakan PJ, Volk TA, Nowak CA, Abrahamson LP (2005) Morphological traits of 30 willow clones and their relationship to biomass production. Can J For Res. 35:421-431.

U.S. Department of Energy (2016) 2016 Billion-Ton Report: Advancing Domestic Resources for a Thriving Bioeconomy, Volume 1: Economic Availability of Feedstocks. M. H. Langholtz, B. J. Stokes, and L. M. Eaton (Leads), ORNL/TM-2016/160. Oak Ridge National Laboratory, Oak Ridge, TN. 448p

Wegman EJ (1990) Hyperdimensional data analysis using parallel coordinates. J Am Stat Assoc. 85:664-675.

Zamora DS, Apostol KG, Wyatt GJ (2014) Biomass production and potential ethanol yields of shrub willow hybrids and native willow accessions after a single 3-year harvest cycle on marginal lands in central Minnesota, USA. Agrofor Syst. 88:593-606.

CHAPTER 5

Differential growth response to fertilization treatments by ten elite shrub willow (*Salix* spp.) bioenergy cultivars

5.1 Abstract

Shrub willow is a strong candidate for use as a dedicated bioenergy crop in moist, temperate climates due to high growth rates, excellent regenerative properties and relatively low nutrient demand. However, large discrepancies exist in the literature as to the benefits of fertilization for improving biomass production. Controlled environment fertilization studies can remove confounding edaphic and climatic factors present in field studies, but their relatively short-term results may not generalize well to harvest rotation lengths of two to four years of this woody, perennial crop. Ten top-performing commercial or pre-commercial cultivars mostly bred in the US were tested for response to five fertilization levels, including a water only control in a greenhouse pot study over a ten-week period. Triploid hybrid *Salix viminalis* × *S. miyabeana* cultivars had the greatest final aboveground biomass. Stem, foliar and total aboveground biomass were significantly correlated with a number of growth and physiological traits. Different strategies for high biomass production among cultivars are discussed in the context of initial growth rates and key leaf traits. Results of this controlled environment experiment are compared directly to measured field performance with a high degree of similarity for most cultivars.

5.2 Introduction

Concerns over the negative impacts of the use of fossil energy sources on the global climate has led to sustained research interests in bioenergy crops. It is expected that in the US the majority of biomass produced for biofuel production with come from dedicated bioenergy crops (U.S. Department of Energy, 2016). The use of shrub willow (*Salix* spp.) as a dedicated bioenergy crop

grown in a short rotation coppice (SRC) systems has been the subject of research in North America and Europe for decades. Shrub willow is characterized by having very high growth rates and adaptability to temperate climates, combined with an ability for rapid regeneration after stems are cutback or coppiced (Karp and Shield, 2008; Kuzovkina and Quigley, 2005).

The *Salix* genus is extremely diverse with at least 300 species that display a remarkable amount of morphological diversity, with growth habits from small shrubs to trees, which can display variability among individuals within a species, as well as interspecific hybridization (Karp et al., 2011; Kuzovkina et al., 2008). Recent studies have demonstrated that novel inter-specific triploid hybrid cultivars can outperform intra-specific diploid and tetraploid cultivars (Fabio et al., 2017a; Fabio et al., 2017b; Serapiglia et al., 2014). The genetic and physiological basis for this trend has just now begun to be studied, but these novel hybrids can display new phenotypes that can be additive or over-dominant with respect to the parents. Future breeding efforts will likely generate large populations of individuals, requiring rapid and effective screening platforms that can predict future performance with reasonable accuracy. Field based systems are under development, but controlled environments still provide an efficient means for measuring growth and physiological traits, except when genotype-by-environment interactions are expected to affect outcomes (Taylor et al., 2016). Greenhouse pot studies provide a controlled environment for measuring differences in genotypes and response to treatments, but it remains challenging to relate these responses to the field setting (Weih and Nordh, 2005), especially considering that SRC willow typically has an optimal age for harvest that is 2-4 years after establishment, when measuring growth and physiological traits can be difficult on large field-grown plants, especially later in the rotation. Identification of plant traits that have the best correlation with biomass production in controlled and field settings will be of most importance.

Research into the yield response of short rotation woody crops to fertilization has produced some conflicting results in a field setting. Stoof et al. (2015) provided a brief overview of research in the northeastern US, while Hangs et al. (2012) provided both European and North American examples that covered not only a wide range of nitrogen (N) fertilization rates, but also species diversity and harvest rotation lengths. When compared to an unfertilized control within each study, both reviews concluded that no clear response was evident. There are a number of edaphic and climatic factors that can affect outcomes, and reducing the confounding factors associated with field conditions may allow for the determination of relationships between plant traits and nutrient uptake and use efficiency. There is evidence that breeding has improved the fertilization response in willow used for biomass production (Aronsson et al., 2014; Weih, 2001). Differences in nutrient use efficiency among cultivars could be exploited for different applications. For instance, if SRC willow is being used for biomass production on marginal agricultural land, then optimal nutrient use efficiency would be a favorable trait. However, if willow are being used as a riparian buffer strip for filtering excess nutrients, then a cultivar with higher rates of luxury nutrient uptake would be optimal. Evaluation in a controlled environment may serve as an efficient selection process for future testing of individuals within a breeding program for various applications (Weih and Nordh, 2002).

In the current study, 10 shrub willow cultivars that differ widely in genetic background and ploidy level were subjected to realistic levels of fertilization in a controlled environment in order to describe differences in growth and physiological traits. These are commercially available or highly selected genotypes that have demonstrated high yields in a field setting, but also differ widely in morphological characteristics. By testing across a range of representative fertilization levels, it can be determined which traits are more influenced by genetics, nutrient

availability, or interactive effects. Traits that are strongly associated with biomass production may help to identity growth strategies that can be predictive of field performance and may aid in accelerating the selection process.

5.3 Materials and methods

5.3.1 Plant material and experimental design

Ten commercial or pre-commercial cultivars mainly from the Cornell University breeding program were selected for a controlled environment experiment (Table 5.1). One-year-old dormant whips were collected in February 2014 from nursery beds at Cornell University's New York State Agricultural Experiment Station in Geneva, NY. Whips were processed into 20-cm cuttings and stored in a freezer at -4^{o}C until planting. On 20 May, 2014, 200 11.4-L plastic pots were filled with a standard peat-based potting mix and arranged on eight benches in a greenhouse. Five cuttings of each of 10 cultivars were randomly assigned to pots separately for each bench. One of five fertilizer treatments was randomly assigned to each of the five cuttings of each cultivar. Benches served as blocks in a two-factorial randomized complete block design, with four replicate blocks for a total of 200 experimental units. Fertilizer treatments were formulated based on the amount of N delivered in a balanced fertilizer solution (Peter's 15-16-17 Peat-Lite Special; Scotts Miracle-Gro Company, Marysville, Ohio, USA). Fertilization treatments were applied weekly at rates 0 (water control), 14, 38, 62 and 86 mg N wk^{-1} delivered in 250 mL of water beginning, on 9 June, 2014, two weeks after the majority of cuttings had broken bud. After four weeks of treatments, the N application rate was increased by a factor of 1.6 for the remaining three weeks of the experiment to compensate for increased biomass accumulation. The total cumulative amounts of N applied by treatment were 0, 123, 334, 546 and

757 mg N over the approximately ten-week experiment. Based on the area of the pot these treatment levels were roughly equivalent to 0, 25, 68, 111 and 154 kg N ha^{-1}.

Table 5.1 Attributes of the 10 cultivars used in the greenhouse study.

Clone ID/ Epithet	Species/Pedigree	Mother	Father	Diversity Group[a]	Sex	Ploidy[b]
'SX61'	*S. miyabeana*	Natural accession		5	F	4X
'Fish Creek'	*S. purpurea*	94006	94001	6a	M	2X
'Fabius'	*S. viminalis* × *S. miyabeana*	SV2	SX67	8	F	3X
'Preble'	*S. viminalis* × *S. miyabeana*	SV2	9970-037	8	F	3X
'Seneca Chief'	*S. viminalis* × *S. miyabeana*	SV7	9970-037	8	F	3X
LA970253	*S. viminalis* × *S. miyabeana*		'Shrubby'	8	F	3X
'Millbrook'	*S. purpurea* × *S. miyabeana*	95026	SX64	9	F	3X
'Victor'	*S. purpurea* × *S. miyabeana*	00-01-088	SX67	9	F	3X
'Geneva Giant'	(*S. koriyanagi* × *S. purpurea*) × *S. miyabeana*	99239-015	SX67	9b	F	3X
'Dimitrios'	(*S. schwerinii* × *S. viminalis*) × *S. aeygyptiaca*	'Tora'		NA[c]	M	3X

[a]Diversity group codes are a direct reference to cultivar species/pedigree.
[b]Ploidy level estimated by flow cytometry (Serapiglia et al. 2015).
[c]No diversiy group has been established.

5.3.2 Growth and physiological measurements

Just prior to the first fertilization treatment, the length of each stem in each pot was measured to the nearest 0.1 cm. These measurements occurred at weekly intervals for a total of eight weeks. Number of stems per plant and weekly total stem length estimates were derived from these measurements for each plant. Simultaneous with weekly stem measurements, four leaves per plant were measured using a Minolta SPAD 502 Chlorophyll Meter (Spectrum Technologies, Aurora, Illinois, USA). SPAD estimates foliar chlorophyll on a unitless greenness index that can be used as a proxy for plant N status. Leaves measured for SPAD were fully expanded and positioned in the upper portion of the canopy in order to capture peak productivity. Therefore, different leaves were measured each week.

At the end of the eighth week of measurements on July 29, 2014, one representative leaf from the upper portion of the canopy of each plant was removed and measured for area (cm^2) using a handheld leaf area scanner model no. CI-203 (CID Bio-Science, Camas, Washington, USA). The same leaves were also measured with the SPAD meter prior to being oven dried at 65^{o}C to a constant weight. The individual leaves were then weighed and specific leaf area (SLA) was calculated as the ratio of leaf area to dry mass ($cm^2\ g^{-1}$). Whole plant aboveground biomass was harvested, partitioned into leaves and stems and weighed fresh. Whole plant total leaf area was measured by scanning each leaf on an LI-3100C leaf area meter (Li-Cor, Lincoln, Nebraska, USA) with conveyor belt. Stems and leaves were placed in a drying oven at 65^{o}C and dried to a constant mass for dry weight determination. Plant total specific leaf area was calculated as the total leaf area of the plant divided by the total dry weight of the leaves. Dried stem and leaf tissues were recombined into a single total aboveground biomass sample for each plant and milled to pass a 1.0 mm mesh. Total C and N were measured as a percent of dry matter using a

LECO TruMac CN Analyzer (Leco Corporation, St. Joseph, MI, USA). Nitrogen use efficiency (NUE) was calculated as total aboveground biomass divided by N content of the biomass (g g^{-1}). Two plants were removed from the experiment by the fifth week of treatments due to abnormally poor growth, resulting in 198 independent experimental units.

5.3.3 Statistical analysis

Weekly leaf SPAD data was analyzed using a repeated measures mixed effects model in PROC MIXED using SAS ver. 9.4 software (SAS Institute Inc., 2013). The effect of cultivar, fertilizer rate and time were considered fixed effects, with time modeled as a continuous variable (i.e. 1 df), while the block effect was considered random. Exploratory plots of the data indicated a curvilinear response over time for the main effects, so a quadratic time effect was added to the model. The HTYPE=1 option was added to the model statement that hypotheses tests were conducted sequentially (Littell et al., 2006). Initial model runs indicated that there were no significant (at the $P \leq 0.05$ level) third-order interactions involving fertilizer, cultivar and time, or quadratic time. Therefore these model effects were removed and the final model took the following form:

$$Y_{ijkl} = \mu + C_i + F_j + CF_{ij} + T_k + CT_{ik} + FT_{jk} + T_k^2 + CT_{ik}^2 + FT_{jk}^2 + B_l + e_{ijkl} \qquad (1)$$

where, Y_{ijkl} is the observed stem length, μ is the overall mean, C_i is the effect of the *i*th cultivar, F_j is the effect of the *j*th fertilizer treatment, T_k is the *k*th time effect in weeks, CT_{ik} is the interaction between cultivar *i* and time *k*, FT_{jk} is the interaction between fertilizer treatment *j* and time *k*, T^2_k is the quadratic time term, CT^2_{ik} is the cultivar-quadratic interaction, FT^2_{jk} is the fertilizer treatment-quadratic interaction, B_l is the random block effect, and e_{ijkl} is the random

error.

A number of variance-covariance structures were tested in order to model the repeated effects with pot serving as the subject, and model fit was assessed using the AIC and BIC. While the unstructured variance-covariance structure provide the best overall fit, the heterogeneous Toeplitz (TOEPH) structure was second best, far fewer variance-covariance parameters to be estimated. The TOEPH variance-covariance structure is well-suited to repeated measures data, because it allows each time period to have a unique variance, and it specifies that adjacent measurements made in time share a specific common correlation coefficient and measurements made further apart in time become less correlated (Littell et al., 2000). Therefore, the more parsimonious TOEPH structure selected as the best variance-covariance structure for the stem length data. Studentized and Pearson residuals plots from the final model were checked for compliance with model assumptions.

For the remaining traits collected just once at the end of the experiment, cultivar and fertilizer rate and their interaction were considered fixed effects, while block was considered random. Residual frequency distributions and plots of residual vs predicted values were used to visually assess model assumptions. Random variance was partitioned between block effect and residual error and each component is presented as a percent of the total. When significant ($P < 0.05$) differences in fixed effects were detected least squared means were calculated and the Tukey HSD test was use for means comparisons. Pearson correlation coefficients were calculated between final biomass estimates and other measured traits using the Multivariate Methods platform in JMP® PRO version 12 (SAS Institute Inc., 2013).

In order to relate the non-destructive SPAD measurements to foliar nitrogen status, the individual leaves collected for SLA were pooled across replicates and analyzed for total C and N

as described above. Leaf samples needed to be pooled to meet the mass requirements of the instrument. The individual leaf SPAD readings were also averaged across replicates and used as the predictor variable against foliar N concentration in a linear regression analysis.

5.3.4 Modeling stem growth

Weekly non-destructive measurements of stem length provided an opportunity to model growth over time by cultivar and N treatment. Using the Nonlinear Modeling platform in JMP, a series of models were tested to find the overall best fit to the data grouped by individual pot for stem length as the response variable and time in days after budbreak as the explanatory variable. Natural log transformed and non-transformed data were analyzed and model fit was assessed by *R*-squared and AIC, where the latter is in the form of smaller is better. The best fitting model for stem length was the three-parameter Gompertz model. This model is asymptotic with the following form:

$$Y = aExp(-Exp(-b(x-c))) \quad (2)$$

where a is an upper asymptote, c is the x-axis value at the inflection point, or the time where the specific growth rate reaches its maximum, b, which for the Gompertz model, is fixed at approximately 37% of the upper asymptote, a. A more interpretable version of maximum growth rate can be calculated as $b \times c/e$, which in this case has the units cm day^{-1}.

In order to analyze differences in model parameters among the treatment groups, model estimates of inflection points, growth rates and upper asymptotes for each experimental unit were collected as a new dataset and were analyzed as a new set of traits (Meredith and Stehman, 1991), using the same mixed model ANOVA described above. These variables were also included in multivariate correlations.

Finally, graphs of absolute growth rate were constructed by plotting the first derivative of

the model predicted stem length vs. days after budbreak. Relative growth rate graphs were constructed by using the natural log transformed stem length data in the modeling step and then taking the first derivative of that function and plotting them against days after budbreak (Paine et al., 2012).

5.4 Results

5.4.1 Growth and physiological traits

The ANOVA *F*-tests for the main effect of cultivar was significant at the $P < 0.0001$ level for all traits except stems per plant, which was significant at the $P < 0.001$ level (Table 5.2). For the main effect of fertilization treatment, 14 of 17 traits were significant at the $P < 0.0001$ level. Two traits unaffected by fertilization were stems per plant and single-leaf SLA, which are likely under strong genetic control. The third trait not significant for fertilization treatment was initial total stem length, because these measurements occurred before the first fertilizer application. The final stem length measurement occurred after seven weeks of fertilization treatments. Six traits showed a significant interaction between fertilizer treatment and cultivar and were mostly leaf traits (Table 5.2). Traits associated with single-leaf SPAD, plant N concentration and N use efficiency where highly significant for both fertilizer treatment and cultivar and showed no significant interaction.

There was no significant interaction between fertilization treatment and cultivar for total aboveground and stem biomass. Both total plant aboveground biomass and stem biomass increased linearly with each fertilization level being significantly different that the others (Figure 5.1a). There were significant differences among cultivars as well, with the top cultivar, LA970253, having significantly greater total plant and stem biomass than seven other cultivars (Figure 1b). The cultivars grouped rather well for total plant biomass according to diversity

group, which represents the cultivar species or species hybrid. The four triploid *S. viminalis* × *S. miyabeana* cultivars had the greatest total biomass, and generally produced more biomass than the three triploid *S. purpurea* × *S. miyabeana* cultivars. The tetraploid *S. miyabeana* check cultivar, 'SX61', and the diploid *S. purpurea* cultivar, 'Fish Creek', had intermediate total biomass.

Table 5.2 Mixed model ANOVA fixed effects *F*-test statistics for single time point growth and physiological traits.

	Fixed effects			**Random effects**	
Trait	**Treatment (df=4)**	**Cultivar (df=9)**	**Interaction (df=36)**	**Block σ^2 (%)**	**Error σ^2 (%)**
Initial stem length	1.04 *ns*	**42.5*** **	1.4 *ns*	24.5	75.5
Final stem length	**13.2*** **	**12.5*** **	1.0 *ns*	9.3	90.7
Stems per plant	1.3 *ns*	**3.8** **	0.8 *ns*	1.2	98.8
Plant moisture content	**338.5*** **	**9.5*** **	**1.6***	19.9	80.1
Plant total aboveground biomass	**135.2*** **	**11.6*** **	1.3 *ns*	16.1	83.9
Stem dry weight	**90.6*** **	**18*** **	1.4 *ns*	21.0	79.0
Leaf dry weight	**210.2*** **	**16.3*** **	**1.7***	7.0	93.0
Stem/leaf biomass ratio	**3.2***	**62.2*** **	**2.0***	33.3	66.7
Plant total leaf area	**215.6*** **	**12*** **	**1.6***	23.5	76.5
Plant total SLA	**123.0*** **	**17.9*** **	1.5 *ns*	38.9	61.1
Single-leaf mass	**24.5*** **	**126.1*** **	**1.7***	12.3	87.7
Single-leaf area	**23.5*** **	**120.5*** **	1.4 *ns*	25.6	74.4
Single-leaf SLA	1.69 *ns*	**14.3*** **	0.9 *ns*	21.3	78.7
Single-leaf SPAD	**85.7*** **	**11.3*** **	**1.6***	6.5	93.5
Plant N concentration	**66.5*** **	**8.2*** **	1.0 *ns*	6.7	93.3
Plant C/N ratio	**90.1*** **	**12.4*** **	1.3 *ns*	10.8	89.2
N use efficiency	**92.0*** **	**12.4*** **	1.3 *ns*	10.1	89.9

***** $P < 0.05$, ****** $P < 0.001$, ******* $P < 0.0001$, *ns* no significant difference.

In contrast to the trend among fertilizer treatments, stem biomass among cultivars did not track total aboveground biomass well. LA970253 had the greatest stem biomass, followed by 'Fish Creek' and 'Fabius'. The cultivar 'Dimitrios' had the lowest stem biomass and the second

lowest total aboveground biomass.

There was a significant interaction between fertilization treatment and cultivar for plant leaf dry biomass and the stem/leaf dry biomass ratio (Table 5.2). For the most part, plant leaf dry biomass increased in linear fashion with increasing fertilization, however changes in rank among cultivars within fertilization levels, especially as lower levels, likely contributed to the significant treatment-by-cultivar interaction (Figure 5.2a).

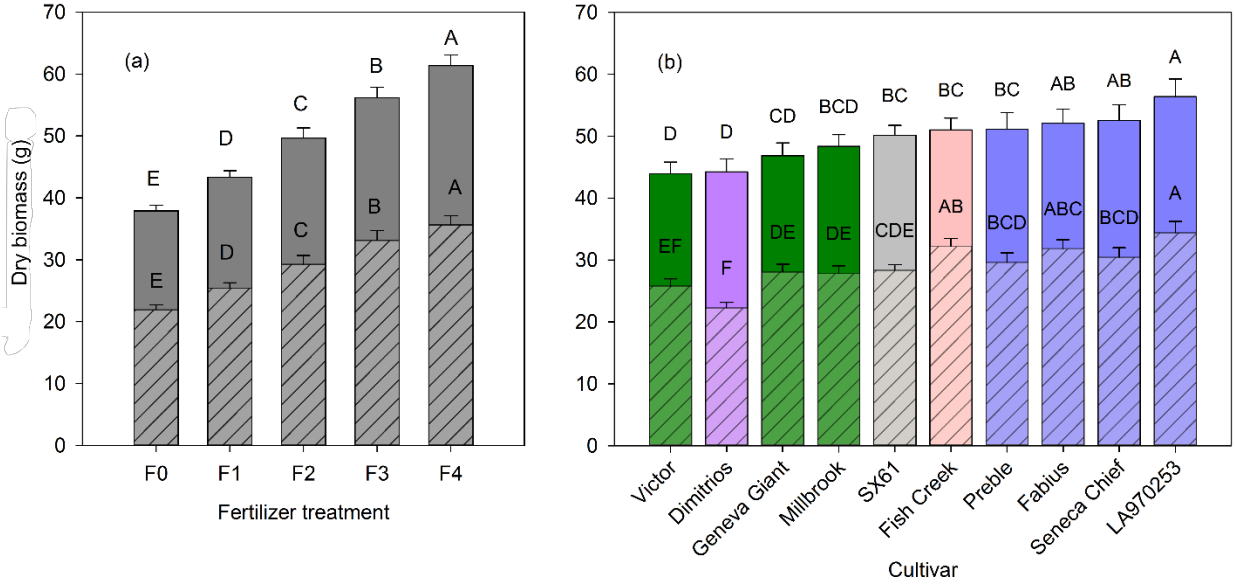


Figure 5.1 Plant total aboveground biomass and stem biomass at the end of the experiment. (a) fertilization treatment; (b) cultivar. Hashed bars represent dry stem biomass, while solid bars represent total aboveground dry biomass. Fertilizer levels correspond to cumulative amounts of 0 (F0), 123 (F1), 334 (F2), 546 (F3) and 757 (F4) mg N. Different letters above bars indicate statistical differences among levels according to Tukey's HSD means comparison.

A slightly curvilinear response in stem/leaf dry biomass ratios across fertilization levels for a number of cultivars likely contributed to the significant interaction for that trait, as there was a sharp decrease in stem/leaf dry biomass ratios at the highest fertilization treatment for a number of cultivars (Figure 5.2b). Other cultivars, including 'Preble', 'Seneca Chief', 'Victor' and 'Millbrook' showed little change across treatments or a slight increase in stem/leaf dry

biomass ratio at the highest fertilization level. Plant total leaf area increased in linear fashion across the five fertilization treatments (Table 5.3). Since leaf dry weight also increased linearly, the differences in plant total SLA were less dramatic. Total SLA increased significantly from treatment F0 to F3, but there was no difference between the F3 and F4 treatment levels.

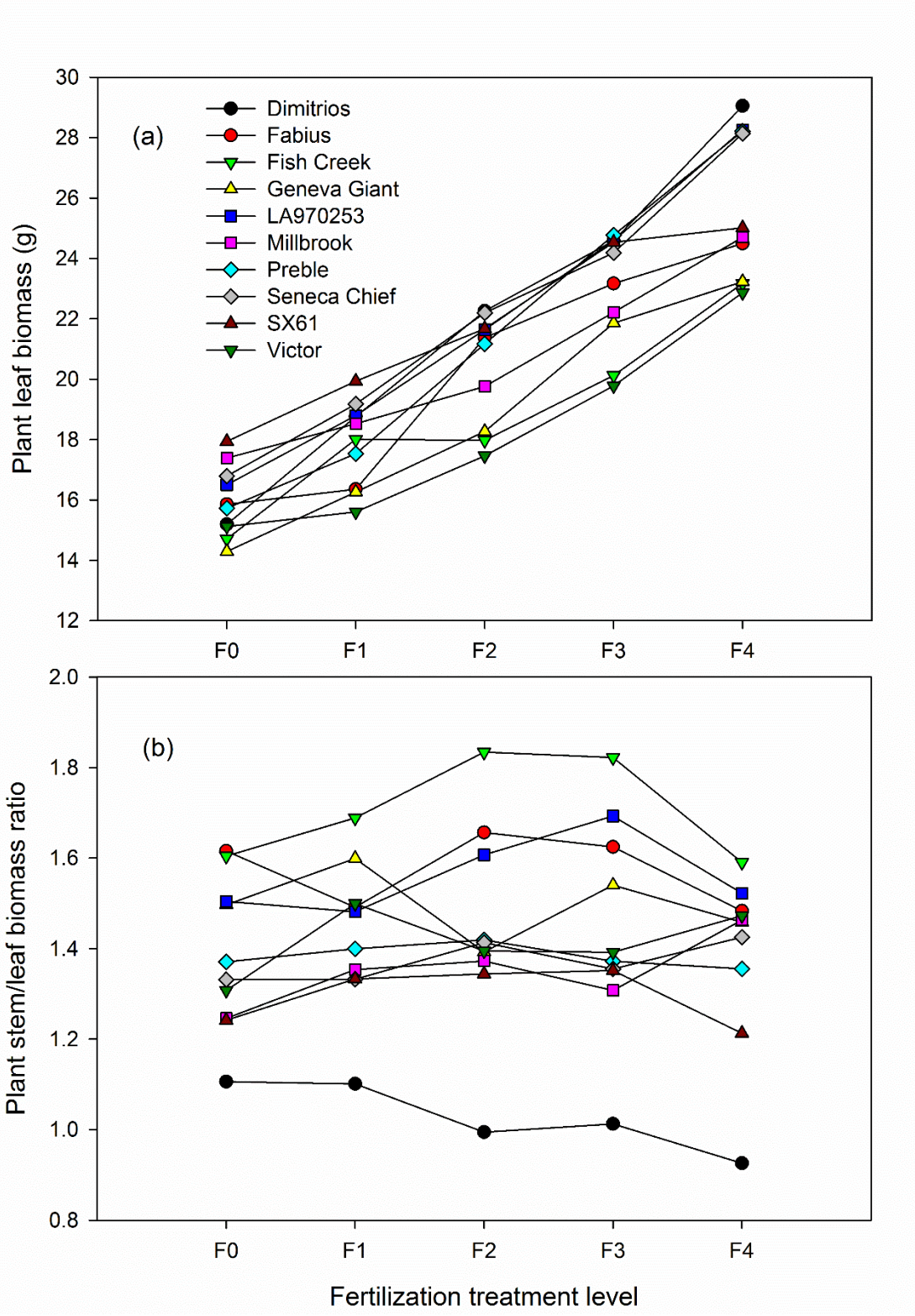

(a)
Dimitrios
Fabius
Fish Creek
Geneva Giant
LA970253
Millbrook
Preble
Seneca Chief
SX61
Victor
Plant leaf biomass (g)
30
28
26
24
22
20
18
16
14
12
F0
F1
F2
F3
F4
(b)
Plant stem/leaf biomass ratio
2.0
1.8
1.6
1.4
1.2
1.0
0.8
F0
F1
F2
F3
F4
Fertilization treatment level

Figure 5.2 Response curves for plant leaf dry biomass (a) and plant stem/leaf dry biomass ratio (b) across fertilization levels.

Table 5.3 Mean values and means separations for fertilization treatments of single-time traits.

Fertilization treatment	Initial total stem length		Final total stem length		Stems per plant		Plant moisture content	
F0	22.5	A	289.5	B	2.6	A	0.58	D
F1	25.1	A	359.2	A	3.0	A	0.61	C
F2	23.9	A	379.4	A	2.9	A	0.64	B
F3	23.9	A	383.0	A	2.8	A	0.66	A
F4	23.6	A	388.1	A	2.7	A	0.67	A

Fertilization treatment	Leaf dry weight		Stem/ leaf biomass ratio		Plant total leaf area		Plant total SLA	
F0	15.9	E	1.38	A	1757.2	E	109.7	D
F1	17.9	D	1.43	A	2203.4	D	122.1	C
F2	20.4	C	1.44	A	2728.0	C	133.8	B
F3	23.0	B	1.45	A	3309.0	B	143.8	A
F4	25.7	A	1.39	A	3785.6	A	146.9	A

Fertilization treatment	Single-leaf mass		Single-leaf area		Single-leaf SLA		Single-leaf SPAD	
F0	0.13	B	22.9	B	176.8	A	30.8	C
F1	0.13	B	22.9	B	180.0	A	30.8	C
F2	0.13	B	24.6	B	185.3	A	35.1	B
F3	0.15	A	28.1	A	182.1	A	40.9	A
F4	0.17	A	30.5	A	180.9	A	42.9	A

Fertilization treatment	Plant N concen-tration		Plant C/N ratio		N use efficiency	
F0	0.73	D	65.3	A	145.1	A
F1	0.75	D	62.0	A	137.3	A
F2	0.93	C	51.0	B	113.0	B
F3	1.22	B	39.5	C	87.9	C
F4	1.37	A	35.0	C	77.8	C

In contrast, when single representative leaves were sampled, only the two highest fertilization levels resulted in significantly greater single-leaf mass and leaf area, and there were no differences in single-leaf SLA, suggesting that increases in mass and area were proportional to one another (Table 5.3). There was no significant difference between the F0 and F1 treatments for plant N concentration, C/N ratio or NUE, while the F3 and F4 treatments had the greatest N concentrations, the lowest C/N ratios and consequently the lowest N use efficiencies among the five treatment levels.

There were significant cultivar effects, including differences in initial total stem length, where the triploid *S. viminalis* × *S. miyabeana* hybrids, LA970253, 'Preble' and 'Fabius' produced the most initial stem length, and 'Dimitrios' and 'SX61' produced the least. There were also strong differences in leaf traits. 'Dimitrios' produced leaves that were significantly greater in mass than all other cultivars and were 1.6-times greater than the next highest cultivar, 'SX61', and 3.6-times greater than the cultivar with the least massive leaves, 'Fish Creek' (Table 5.4). Similarly 'Dimitrios' had a leaf area estimate 1.9-times greater than 'SX61' and 3.8-times greater than 'Fish Creek'. 'Dimitrios' also had the lowest stem/leaf biomass ratio, suggesting a heavy investment in large leaves at the expensive of stem growth. While 'Dimitrios' and 'Fish Creek' were at the opposite extremes for leaf mass and area and stem/leaf biomass ratio, they both had similarly high SPAD readings. The LA970253 cultivar produced the greatest total aboveground and stem biomass, the greatest single-leaf SLA and the lowest SPAD reading and plant N concentration, so not surprisingly it also had the greatest N use efficiency (Table 5.4). 'Seneca Chief' produced the greatest leaf dry biomass and had the second lowest SPAD reading, and along with LA970253' and 'Fabius', had the greatest N use efficiency.

Table 5.4 Mean values and means separations for genotypes of single-time traits.

Genotype	Initial total stem length		Final total stem length		Stems per plant		Plant moisture content	
Dimitrios	8.98	F	210.5	B	2.2	C	0.62	C
Fabius	30.36	AB	416.1	A	2.9	ABC	0.63	BC
Fish Creek	22.23	CDE	373.2	A	2.7	ABC	0.62	C
Geneva Giant	24.56	CD	385.8	A	3.1	AB	0.63	BC
LA970253	34.4	A	360.0	A	2.5	BC	0.64	AB
Millbrook	17.17	E	367.3	A	2.5	BC	0.63	C
Preble	34.80	A	359.5	A	3.4	A	0.63	BC
Seneca Chief	27.82	BC	369.3	A	3.0	AB	0.63	C
SX61	20.2	DE	365.6	A	2.7	ABC	0.65	A
Victor	17.34	E	391.1	A	2.9	ABC	0.62	C

Genotype	Leaf dry weight		Stem/ leaf biomass ratio		Plant total leaf area		Plant total SLA	
Dimitrios	22.0	AB	1.03	F	2897.8	AB	128.8	DE
Fabius	20.2	BC	1.58	B	2448.1	CD	118.9	F
Fish Creek	18.8	CD	1.71	A	2691.8	BC	140.2	ABC
Geneva Giant	18.7	CD	1.49	BC	2327.2	D	120.1	EF
LA970253	22.0	AB	1.56	B	2877.6	AB	128.0	DE
Millbrook	20.5	AB	1.35	DE	2917.4	AB	141.1	AB
Preble	21.5	AB	1.38	DE	3158.2	A	142.9	A
Seneca Chief	22.1	A	1.37	DE	2991.1	AB	133.3	BCD
SX61	21.8	AB	1.30	E	2826.7	AB	127.6	DEF
Victor	18.2	D	1.41	CD	2430.5	CD	131.5	CD

Table 5.4 (continued)

Genotype	Single-leaf mass		Single-leaf area		Single-leaf SLA		Single-leaf SPAD	
Dimitrios	0.29	A	53.5	A	183.5	AB	40.2	AB
Fabius	0.11	E	18.0	D	167.3	C	37.8	ABC
Fish Creek	0.08	F	14.1	D	177.7	BC	40.6	A
Geneva Giant	0.11	DE	18.4	D	162.6	C	37.2	ABCD
LA970253	0.12	CDE	23.4	C	195.7	A	32.3	F
Millbrook	0.14	C	26.7	BC	192.1	AB	33.7	DEF
Preble	0.13	CD	26.4	BC	193.7	A	36.3	BCDE
Seneca Chief	0.13	CD	25.0	BC	187.2	AB	32.7	EF
SX61	0.18	B	28.9	B	163.5	C	35.6	CDEF
Victor	0.13	CDE	23.8	C	186.9	AB	34.9	CDEF

Genotype	Plant N concen-tration		Plant C/N ratio		N use effici-ency	
Dimitrios	1.12	AB	42.8	C	95.0	C
Fabius	0.83	D	59.1	A	131.0	A
Fish Creek	0.93	BCD	55.2	AB	122.2	AB
Geneva Giant	1.20	A	43.3	C	96.4	C
LA970253	0.80	D	60.2	A	133.4	A
Millbrook	0.96	BCD	51.6	ABC	114.0	ABC
Preble	1.07	ABC	44.9	C	99.8	C
Seneca Chief	0.86	CD	57.8	A	128.1	A
SX61	1.07	ABC	46.5	BC	103.5	BC
Victor	1.14	AB	44.3	C	98.6	C

In contrast, ‘Fabius’ had relatively low single-leaf and plant total SLA, and the second greatest stem/leaf biomass ratio. This along with an above average SPAD reading, but the second lowest plant N content, suggests an investment in efficient leaves.

5.4.2 SPAD measurements

Single-leaf SPAD measurements coupled with foliar N concentration data allowed for the development of a calibration dataset for the nondestructive estimation of leaf N from SPAD readings. There was a reasonably strong positive correlation with SPAD explaining

about 68% of the variance in foliar N concentration (Figure 5.3).

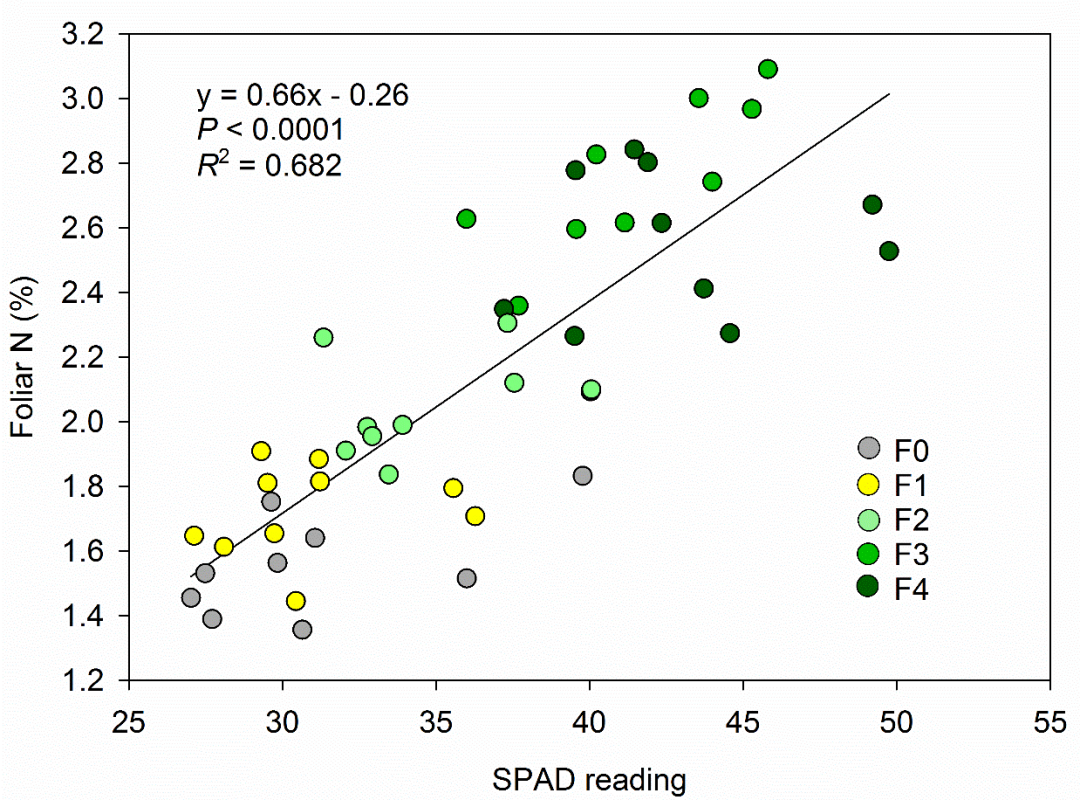


Figure 5.3 Calibration of SPAD readings for predicting foliar N concentration.

Weekly SPAD measurements showed a curvilinear response over time, and the final model contained no third-order interactions. Both the linear and quadratic effects of time were highly significant (Table 5.5), indicating that modeling SPAD response across time as a polynomial function was justified. Initial cultivar SPAD values tended to be greatest for *S. purpurea* and *S. viminalis* × *S. miyabeana* hybrids, while initial SPAD values for *S. purpurea* × *S. miyabeana* cultivars were among the lowest (Figure 5.4a). 'Fish Creek' had the greatest stem/leaf dry biomass ratio and the greatest SPAD readings, which implies that this cultivar has a strategy of concentrating photosynthetic capacity into a relatively small canopy, but it may also have a high N demand. Interestingly, the SPAD values for *S. miyabeana* cultivar 'SX61' were intermediate

between *S. purpurea* ‘Fish Creek’ and the hybrid cultivars, which could suggest a non-additive inheritance pattern for this trait.

Table 5.5 SPAD measurement repeated measures quadratic polynomial analysis of variance table.

Effect	DF	*F*-value	*P*-value
Treatment	4	82.9	**<0.0001**
Cultivar	9	95.98	**<0.0001**
Trmt*cultivar	36	1.97	**0.002**
Time	1	737.59	**<0.0001**
Trmt*Time	4	92.16	**<0.0001**
Cultivar*Time	9	21.75	**<0.0001**
Time^2	1	1002.61	**<0.0001**
Trmt*Time^2	4	2.35	0.056
Cultivar*Time^2	9	16.54	**<0.0001**

SPAD generally increased until between 30 and 40 days after budbreak, with a constant decline until the end of the experiment. The interaction between cultivar and the quadratic effect of time was highly significant, indicating that there were significant differences in the rate of decline among cultivars. In fact, the three cultivars that obtained the greatest SPAD readings had the steepest decline; however, their mean SPAD values remained high for the duration of the experiment. ‘Preble’ in contrast had relatively stable SPAD readings throughout the experiment. ‘Dimitrios’ had the lowest initial SPAD and a shallow rise over time, but ended with the second greatest SPAD value at the final time point. This cultivar also had the second lowest final total biomass (Figure 5.1) and a stem/leaf dry biomass ratio far lower than all other cultivars (Figure 5.2). These cultivar-specific deviations from the general trend likely contributed the most to the significant cultivar-by-time linear and quadratic effects. However, the general downward trajectory in the second half of the experiment may indicate a dilution of N as biomass increased, but it may also indicate that N supply was not keeping up with demand.

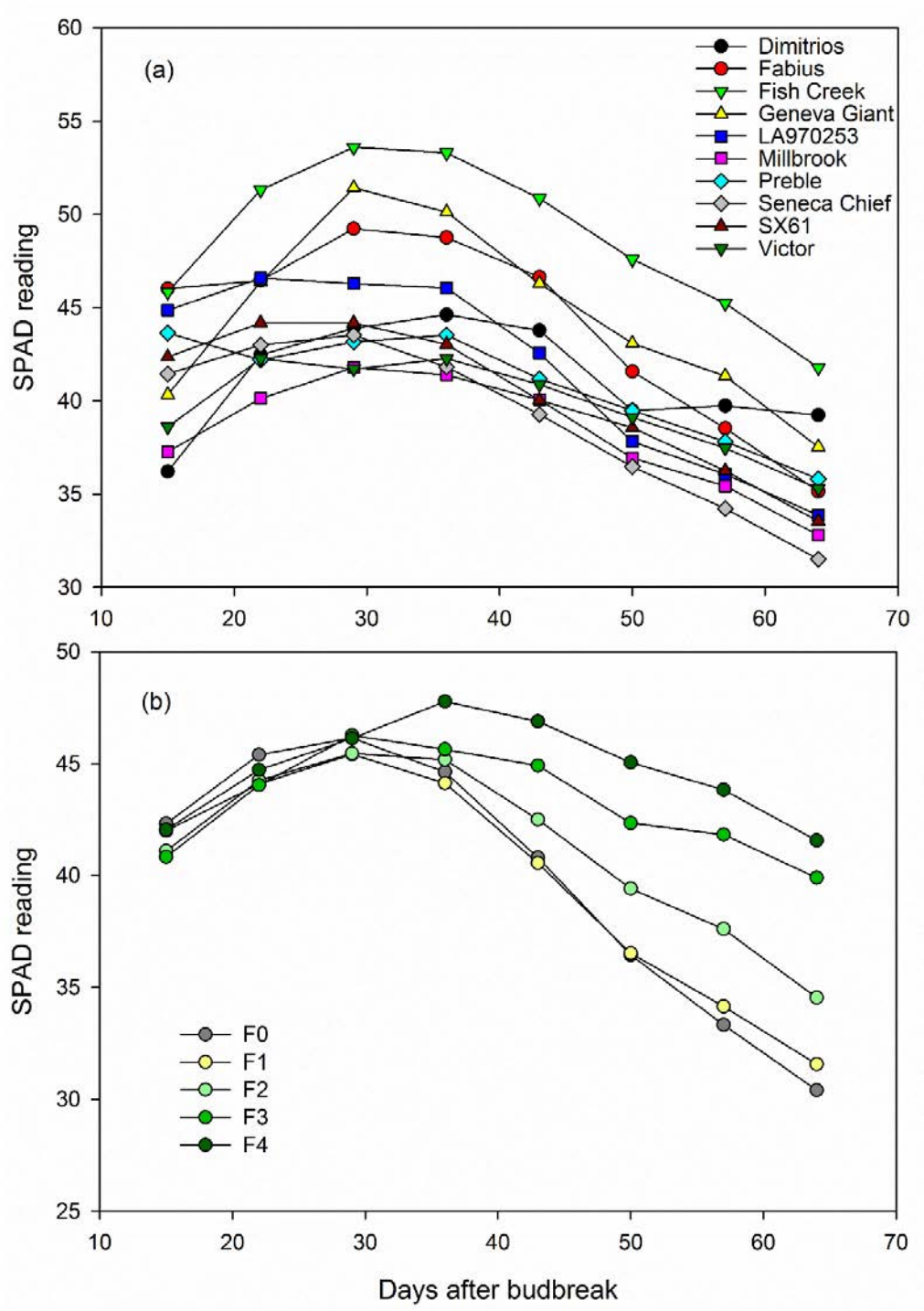


Figure 5.4 SPAD readings over time. (a) 10 cultivars; (b) five fertilization levels. The interaction terms between cultivar and both the linear and quadratic time effects are significant, but only the linear time-by-treatment interaction was significant.

For fertilization treatments, SPAD readings were similar for the first three time points, but by the second half of the experiment separation in SPAD among the treatment levels became increasingly pronounced in expected fashion, although the water-only control and the first treatment level tracked together for the most part (Figure 5.4b). The interaction between treatment and the quadratic effect of time was not significant, indicating that the downward trend in SPAD reading across time did not differ among the five treatment levels.

5.4.3 Modeling stem growth

Analysis of variance of growth modeling parameters showed that the main effects of fertilizer treatment and cultivar were significant for all three of the Gompertz modelling parameters, while the interaction was not significant for any parameter (Table 5.6). The water-only control F0 and F1 treatments reached the inflection point earliest at 30 and 33 d after budbreak, respectively, and were significantly different from one another and from the other three treatment levels, according to the means separation test (Table 5.7, Figure 5.5a). Levels F2, F3 and F4 were not significantly different from one another and ranged from 36 to 37 d after budbreak, suggesting that fertilizer treatments extended the period of accelerated growth. While maximum growth rate was greatest in the F4 treatment at 10.2 cm d^{-1}, it was not significantly different from the other fertilization treatments, except for the water control, which was 8.6 cm d^{-1}. Fertilization also affected the modeled asymptotes, where the F0 and F1 treatments were significantly lower than the three higher treatment levels and were also significantly different from one another at 320 and 417 cm, respectively. The F2, F3 and F4 levels ranged from 474 to 495 cm, but did not differ significantly from each other.

Table 5.6 Mixed effects ANOVA of growth modeling parameters from the Gompertz modeling of each pot using weekly stem length data as the response variable.

	Fixed effects			Random effects	
Growth Parameter	Treatment (df=4)	Cultivar (df=9)	Interaction (df=36)	Block σ^2 (%)	Error σ^2 (%)
Inflection point	42.1***	25.8***	0.98	12.4	87.6
Growth rate	3.58*	12.8***	0.91	7.5	92.5
Asymptote	38.7***	17.8***	1.2	7.7	92.3

***** $P < 0.05$, ****** $P < 0.001$, ******* $P < 0.0001$

There was a gradient in inflection points among the 10 cultivars, with those that achieved the greatest final biomass having the earliest inflection points, and those with the lowest total biomass having the latest inflection points (Table 5.7, Figure 5.5b). The main effect of cultivar was also highly significant for maximum growth rate. ‘Dimitrios’ was significantly lower than all other cultivars, but interestingly LA970253, ‘Preble’ and ‘Seneca Chief’ were the next lowest ranked cultivars for maximum growth rate, although they were only statistically significantly lower than the cultivar with the greatest growth rate, ‘Fabius’. There were strong differences in modeled asymptotes among the 10 cultivars, however their rankings were not necessarily as expected based on final aboveground dry biomass rankings. ‘Fish Creek’ and ‘Victor’ appeared to have a slight increase in growth rate at the end of the experiment (Figure 5.5b), which caused them to have the greatest modeled asymptote, although they were only significantly greater than the bottom three cultivars (Table 5.7). Interestingly LA970253 had the second lowest asymptote, but the greatest overall aboveground biomass (Figure 5.1b). While the data overall have a sigmoidal shape, some cultivars may not have slowed down growth by the end of the experiment.

Table 5.7 Mean values and separation tests for stem length growth modeling parameters**.**

Treatment	Inflection point (days)		Maximum growth rate (cm day^{-1})		Asymptote (cm)	
F0	30.5	C	8.6	B	320.1	C
F1	33.1	B	9.8	AB	417.2	B
F2	36.4	A	9.9	A	474.7	A
F3	37.4	A	9.9	AB	495.9	A
F4	36.9	A	10.2	A	495.2	A
Cultivar	**Inflection point (days)**		**Maximum growth rate (cm day^{-1})**		**Asymptote (cm)**	
Dimitrios	37.7	AB	5.6	C	267.6	E
Fabius	31.7	DE	11.8	A	484.1	ABC
Fish Creek	38.7	AB	10.1	AB	513.6	A
Geneva Giant	34.3	CD	10.6	AB	466.4	ABCD
LA970253	29.7	E	9.2	B	405.5	D
Millbrook	36.5	BC	10.1	AB	448.7	ABCD
Preble	31.8	DE	9.2	B	433.5	BCD
Seneca Chief	34.1	CD	9.5	B	453.2	ABCD
SX61	34.4	CD	10.1	AB	424.6	CD
Victor	39.6	A	10.6	AB	508.8	AB

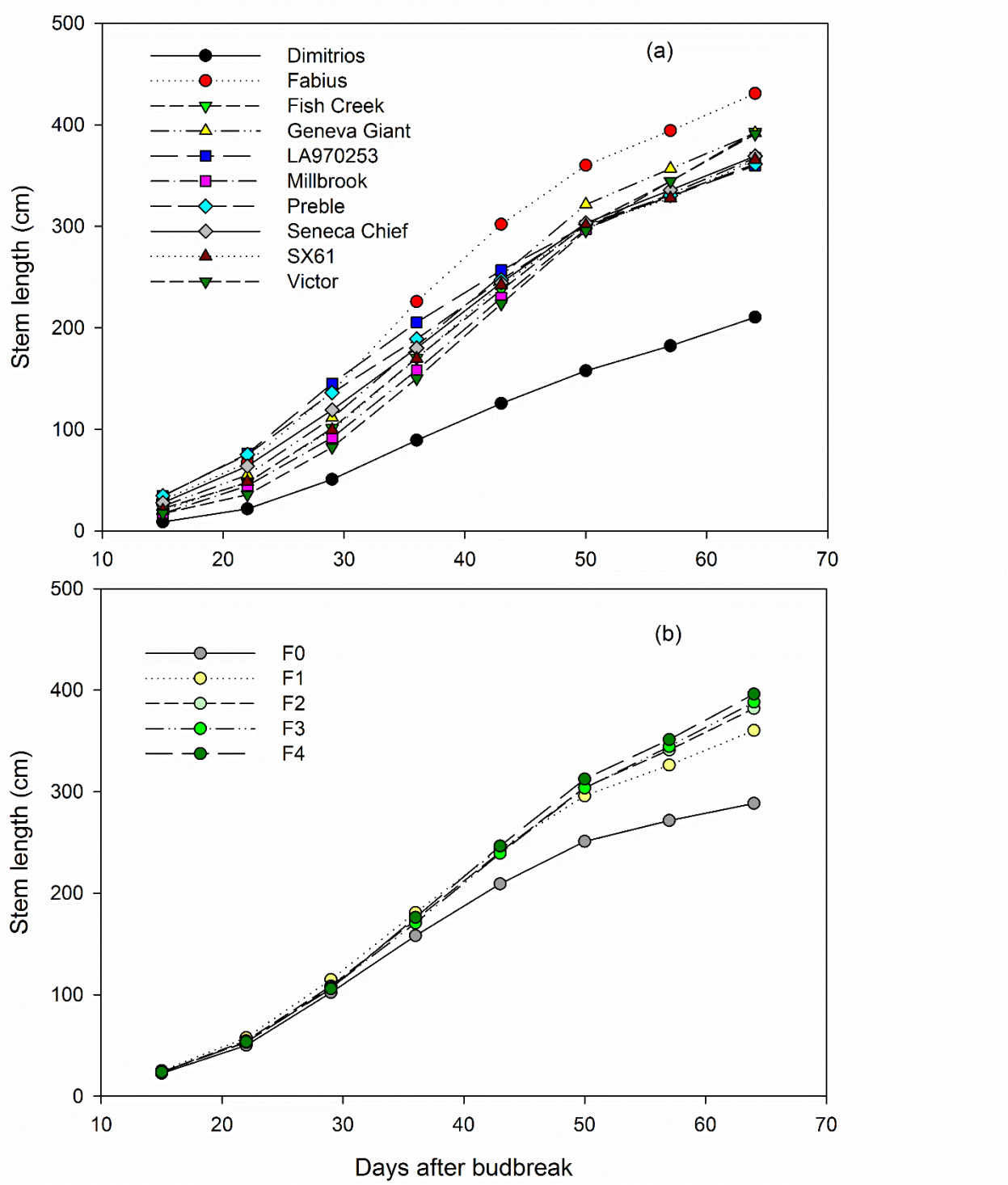


Figure 5.5 Total stem length measurements over time. (a) 10 cultivars; (b) five fertilization levels.

Modeling absolute growth rate over the course of the experiment may have helped to

explain some of the above mentioned patterns of stem length growth over time (Figure 5.6). ‘Fabius’ had very rapid early growth and seemed to respond to fertilization with the greatest peak in absolute growth rate. ‘Preble’ and ‘Seneca Chief’ also had peaks at the highest level of fertilization. While all the water-only control treatment produced the lowest curve for nearly all cultivars, some experienced their peak absolute growth rates at lower fertilization levels, such as ‘Fish Creek’ and ‘Millbrook’. If mean total final aboveground dry biomass for each cultivar was regressed on mean initial total stem data separately for each fertilization treatment, between 56% and 71% of variation in total biomass can be explained for the F2, F3 and F4 treatments (Figure 5.7). However, there is no significant relationship between these two variables at the F0 and F1 levels.

Pairwise correlations among all the measured traits showed that total aboveground dry biomass was highly, positively correlated with plant total leaf area (r = 0.86, Table 5.8), which was greater than the correlation between aboveground biomass and plant total SLA (r = 0.67). Among the single-leaf traits, SPAD had a significant correlation with aboveground biomass (0.51), but not single-leaf area or SLA. For stem biomass, plant total leaf area and plant total SLA had weaker, but still significant correlations (r = 0.73 and r = 0.58, respectively). Single-leaf SPAD was also positively correlated with stem biomass (r = 0.44), but to a lesser degree than with total dry aboveground biomass. The modeled Gompertz asymptote was slightly better correlated with aboveground dry biomass than the measured final total stem length (r = 0.58 vs 0.53). The Gompertz growth rate was not well correlated with aboveground dry biomass (r = 0.34), but it was slightly better correlated with stem dry biomass (r = 0.42). The Gompertz growth rate was also highly correlated with final total stem length (r = 0.93). The Gompertz inflection point was only weakly correlated with aboveground dry biomass (r = 0.19, $P < 0.05$).

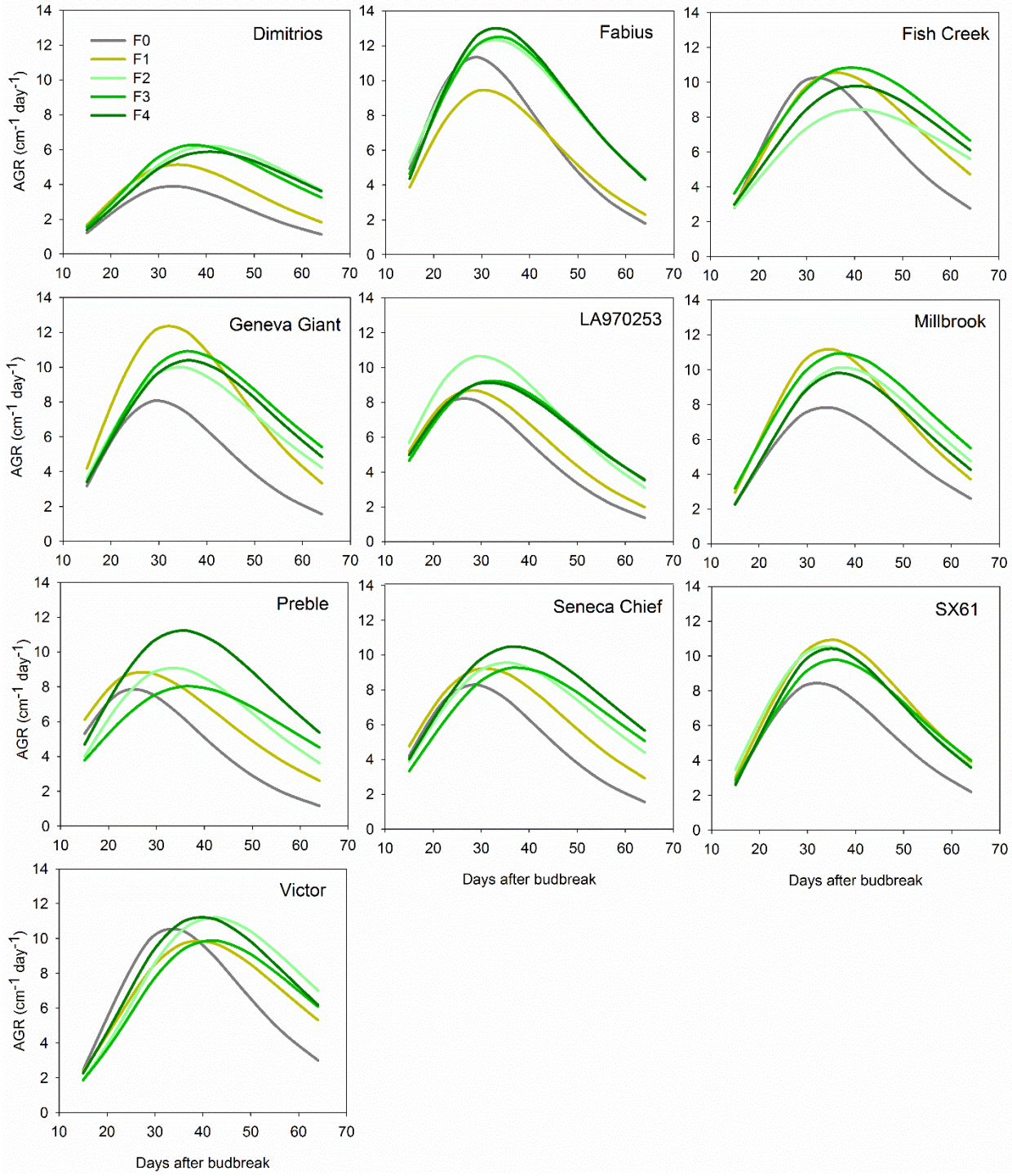


Figure 5.6 Absolute growth rate modeling using the Gompertz parameter estimates for the 10 cultivars at five fertilization levels.

Table 5.8 Pearson's correlation coefficient matrix for 20 traits.

	Initial stem length	Final stem length	Stems per plant	Plant moisture content	Total abvgrd biomass	Stem dry weight	Leaf dry weight	Stem/leaf ratio	Plant total leaf area	Plant total SLA
Initial stem length		0.49	0.46	0	0.3	0.4	0.08	0.49	0.01	-0.1
Final stem length	0.49		0.66	0.39	0.53	0.61	0.31	0.53	0.33	0.33
Stems per plant	0.46	0.66		0	0.07	0.09	0.02	0.12	0.03	0.05
Plant moisture content	0	0.39	0		0.77	0.68	0.81	0.04	0.83	0.75
Total abvgrd biomass	0.3	0.53	0.07	0.77		0.97	0.89	0.35	0.86	0.67
Stem dry weight	0.4	0.61	0.09	0.68	0.97		0.75	0.58	0.73	0.58
Leaf dry weight	0.08	0.31	0.02	0.81	0.89	0.75		-0.09	0.95	0.7
Stem/leaf ratio	0.49	0.53	0.12	0.04	0.35	0.58	-0.09		-0.05	0.04
Plant total leaf area	0.01	0.33	0.03	0.83	0.86	0.73	0.95	-0.05		0.88
Plant total SLA	-0.1	0.33	0.05	0.75	0.67	0.58	0.7	0.04	0.88	
Single-leaf mass	-0.51	-0.46	-0.34	0.23	0.06	-0.14	0.39	-0.67	0.32	0.15
Single-leaf area	-0.51	-0.46	-0.34	0.25	0.08	-0.11	0.4	-0.66	0.37	0.24
Single-leaf SLA	-0.09	-0.11	-0.12	0.13	0.09	0.07	0.11	-0.06	0.24	0.4
Single-leaf SPAD	-0.21	0.09	-0.18	0.64	0.51	0.44	0.54	0.02	0.6	0.58
Plant N concentration	-0.18	0.07	-0.01	0.59	0.31	0.19	0.47	-0.28	0.49	0.43
Plant C/N ratio	0.14	-0.1	-0.01	-0.61	-0.38	-0.26	-0.53	0.28	-0.53	-0.44
N use efficiency	0.15	-0.1	-0.01	-0.62	-0.38	-0.26	-0.53	0.28	-0.53	-0.45
Gompertz inflection pt.	-0.58	0.02	-0.22	0.43	0.19	0.13	0.28	-0.12	0.41	0.56
Gompertz growth rate	0.4	0.93	0.7	0.22	0.34	0.42	0.15	0.45	0.16	0.16
Gompertz asymptote	0.34	0.93	0.53	0.5	0.58	0.64	0.38	0.5	0.45	0.51

Correlation coefficients below 0.14 are not significant at $P = 0.05$ level

Table 5.8 (continued)

	Single-leaf mass	Single-leaf area	Single-leaf SLA	Single-leaf SPAD	Plant N conc.	Plant C/N ratio	N use efficiency	Gomp inflection pt.	Gomp growth rate	Gomp asymptote
Initial stem length	-0.51	-0.51	-0.09	-0.21	-0.18	0.14	0.15	-0.58	0.4	0.34
Final stem length	-0.46	-0.46	-0.11	0.09	0.07	-0.1	-0.1	0.02	0.93	0.93
Stems per plant	-0.34	-0.34	-0.12	-0.18	-0.01	-0.01	-0.01	-0.22	0.7	0.53
Plant moisture content	0.23	0.25	0.13	0.64	0.59	-0.61	-0.62	0.43	0.22	0.5
Total abvgrd biomass	0.06	0.08	0.09	0.51	0.31	-0.38	-0.38	0.19	0.34	0.58
Stem dry weight	-0.14	-0.11	0.07	0.44	0.19	-0.26	-0.26	0.13	0.42	0.64
Leaf dry weight	0.39	0.4	0.11	0.54	0.47	-0.53	-0.53	0.28	0.15	0.38
Stem/leaf ratio	-0.67	-0.66	-0.06	0.02	-0.28	0.28	0.28	-0.12	0.45	0.5
Plant total leaf area	0.32	0.37	0.24	0.6	0.49	-0.53	-0.53	0.41	0.16	0.45
Plant total SLA	0.15	0.24	0.4	0.58	0.43	-0.44	-0.45	0.56	0.16	0.51
Single-leaf mass		0.97	0.02	0.3	0.31	-0.35	-0.35	0.26	-0.45	-0.39
Single-leaf area	0.97		0.25	0.31	0.28	-0.32	-0.32	0.27	-0.47	-0.38
Single-leaf SLA	0.02	0.25		0.07	-0.06	0.09	0.09	0.11	-0.16	-0.05
Single-leaf SPAD	0.3	0.31	0.07		0.58	-0.57	-0.57	0.5	-0.04	0.25
Plant N concentration	0.31	0.28	-0.06	0.58		-0.93	-0.93	0.41	0	0.19
Plant C/N ratio	-0.35	-0.32	0.09	-0.57	-0.93		1	-0.44	0	-0.24
N use efficiency	-0.35	-0.32	0.09	-0.57	-0.93	1		-0.44	0	-0.24
Gompertz inflection pt.	0.26	0.27	0.11	0.5	0.41	-0.44	-0.44		-0.13	0.35
Gompertz growth rate	-0.45	-0.47	-0.16	-0.04	0	0	0	-0.13		0.79
Gompertz asymptote	-0.39	-0.38	-0.05	0.25	0.19	-0.24	-0.24	0.35	0.79	

Correlation coefficients below 0.14 are not significant at $P = 0.05$ level

The greenhouse trial biomass data were also compared to the results from four recent yield trials conducted in the field in Escanaba, MI, Fredonia, NY and Rock Springs, PA, USA (Fabio et al., 2017). The rankings were fairly similar, with LA970253 having top biomass production in the greenhouse trial and the top yield in the yield trials (Table 5.9).

Table 5.9 Comparison of biomass production and cultivar ranking between yield trials and the current greenhouse trial

	Biomass			Rank		
Cultivar	Yield trial yield (Mg ha^{-1} yr^{-1})*	GH total biomass (kg m^{-2})†	GH stem biomass (kg m^{-2})	Field trial yield	GH total biomass	GH stem biomass
LA970253	8.01	1.15	0.70	1	1	1
Victor	7.91	0.90	0.53	2	10	9
Fabius	7.36	1.06	0.65	3	3	3
Preble	7.28	1.04	0.60	4	4	5
Geneva Giant	7.12	0.95	0.57	5	8	7
Millbrook	6.98	0.99	0.57	6	7	8
Seneca Chief	6.69	1.07	0.62	7	2	4
Fish Creek	6.26	1.04	0.66	8	5	2
SX61	6.06	1.02	0.58	9	6	6
Dimitrios	5.32	0.90	0.45	10	9	10

*Annualized yields are based on the mean of four yield trials from the first three-year rotation. See Fabio et al. (2017) for trial details.
†GH, greenhouse trial total and stem biomass from the current study. Biomass production is based on dry matter over the area of the pot.

'Fabius' was ranked third and 'Preble' ranked fourth in both field greenhouse trials. 'Dimitrios' was ranked last in the yield trials, second-to-last in greenhouse total dry biomass, and last in greenhouse stem dry biomass. The largest discrepancy in ranking was for 'Victor' which was ranked last for total dry biomass in the greenhouse trial, but ranked second in the yield trials. If this cultivar is removed from the datasets, the resulting correlation coefficients are relatively high between field trial yield and total aboveground dry biomass (r = 0.69) and stem dry biomass (r =

0.74) from the greenhouse trial.

5.5 Discussion

Discerning patterns emerged in growth and physiological traits among the cultivars tested in this controlled environment experiment. In general, the triploid *S. viminalis* × *S. miyabeana* cultivars obtained the greatest total aboveground dry biomass and greatest stem dry biomass in this greenhouse trial. This was also the case in another pot study involving *S. viminalis* × *S. miyabeana* genotypes from the US breeding program (Hangs et al., 2011). Bouman and Sylliboy (2012) measured first-year growth in the field in eastern Canada and found that 'Fabius' had greater aboveground biomass production compared to a number of native and cultivated diploid and tetraploid species. One main objective of this research was to identify traits that might be associated with particular growth strategies that lead to greater biomass production. It has been observed that in controlled environment trials using European genotypes, earlier budbreak and rapid initial growth can lead to greater biomass accumulation (Brereton et al., 2013; Rönnberg-Wästljung and Gullberg, 1999; Weih, 2009). The date of budbreak was not recorded for each individual cutting in this trial, but strong differences were measured in initial total stem length just prior to fertilizer applications. Initial stem length explained a significant amount of variation in final biomass production, but only at higher fertilization levels (Figure 5.7), suggesting that without adequate nutrient availability the growth advantage conferred by early initial stem growth cannot be realized.

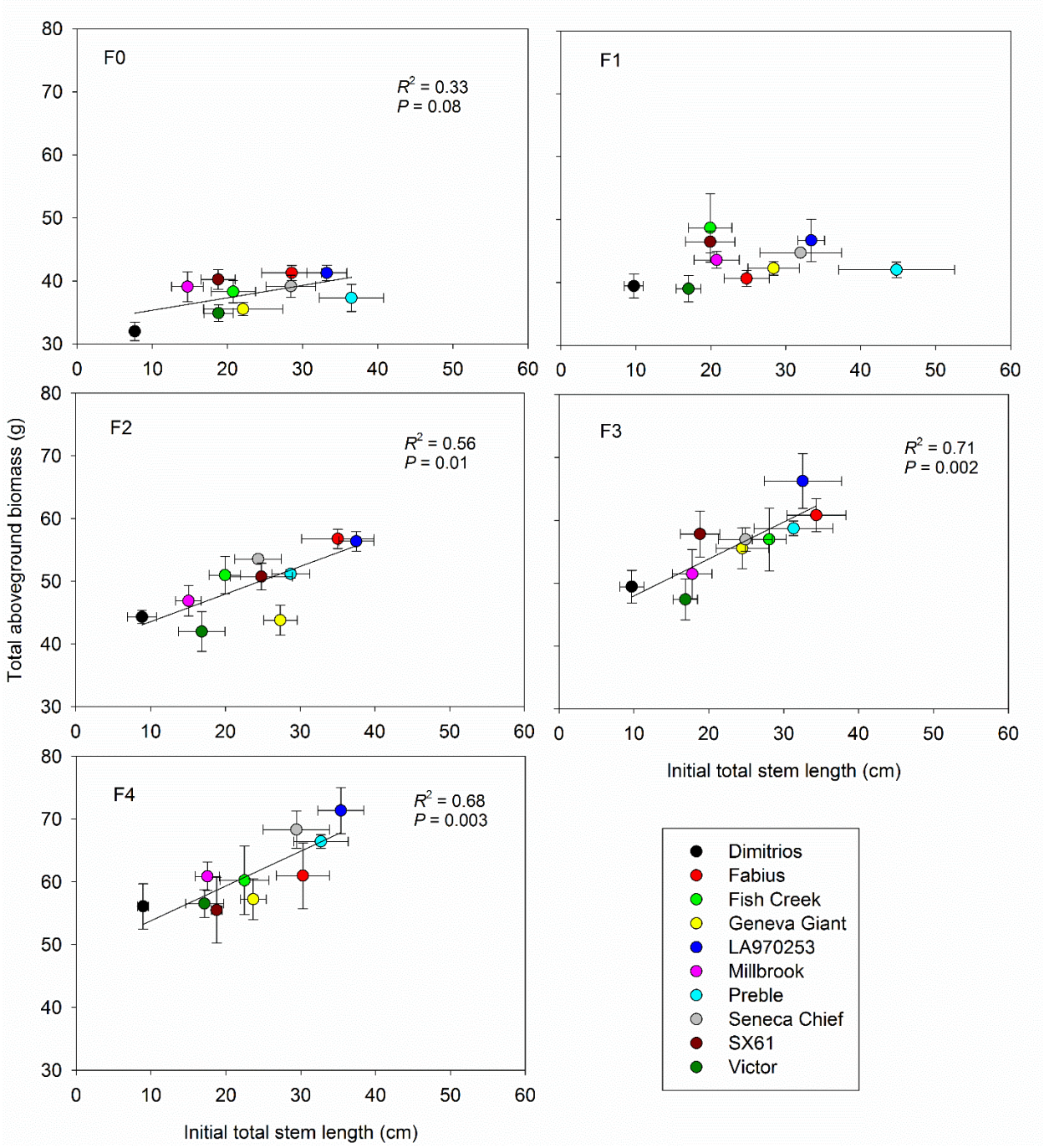


Figure 5.7 Total aboveground biomass vs. initial total stem length for each of the five fertilization treatment levels (F0 - F4). Lines, R^2 and *P*-values are from linear regressions performed within each treatment level. Vertical and horizontal bars represent standard error (n = 4) for total aboveground biomass and initial total stem length, respectively.

The growth modeling data show that the control F0 and the lowest F1 fertilization treatment had inflection points that were significantly earlier in time, the lowest maximum growth rates, and significantly lower final asymptotes than the higher fertilization levels. Inadequate or low nutrition resulted in shorter stems that reached a lower maximum growth at an earlier point in time.

Interestingly, the top-yielding cultivar in the trial was LA970253, but it also had the earliest inflection point, the second lowest maximum stem growth rate, and the second lowest final asymptote. In complete contrast 'Victor' had the latest infection point and the second greatest asymptote, but it had the lowest total aboveground biomass. Perhaps stem length is not the best indicator of aboveground biomass production, because it may not capture stem radial growth. Greater allocation towards stem biomass could potentially benefit yield, as shrub willow is typically harvested during winter dormancy after leaves have been shed.

There was a strong positive correlation (r = 0.86) between plant total leaf area and total aboveground biomass, which has been observed in other willow pot studies (Andralojc et al., 2014; Mamashita et al., 2015; Weih and Nordh, 2005). Whole canopy leaf traits, such as plant dry leaf weight, total leaf area and total SLA were positively correlated with plant N concentration and leaf SPAD reading, and negatively correlated with N use efficiency. Not surprisingly, the top biomass producers also had the greatest N use efficiencies. LA970253 also had the greatest single-leaf SLA, the lowest single-leaf SPAD reading, and the greatest plant N use efficiency. Considering this and the cultivar's high biomass production capability, it may have a strategy of low photosynthetic investment in individual leaves, but it may compensate by having greater leaf area (Tharakan et al., 2005). 'Seneca Chief' achieved the second greatest total aboveground dry biomass, but it had the greatest leaf dry weight and a relatively low stem/leaf

biomass ratio, compared to LA970253 and ‘Fabius’, which had the third greatest biomass production, but greater stem biomass and a similar stem/leaf biomass ratio compared to LA790253. ‘Fabius’ also the lowest plant total SLA and significantly greater leaf SPAD reading than ‘Seneca Chief’ and LA970253, which suggests a strategy of high biomass production through a heavier investment in photosynthetic capacity in the leaves.

A key consideration is whether early growth measurements can predict expected field grown performance of cultivars at harvest age. When compared to results of field trials, the performance of the cultivars in this greenhouse trial were similar with a few exceptions, despite the large difference in the length of time between planting and harvesting. If the cultivar ‘Victor’ is removed from the datasets, the resulting correlation coefficients are relatively high for total aboveground dry biomass ($r = 0.69$) and stem dry biomass ($r = 0.74$). This cultivar did have the latest inflection point, but it also has the second highest modeled asymptote and the second greatest measured final total stem length. It was assumed that yields of ‘Dimitrios’ in the field trials were low because of herbivory and pest damage, which was frequently observed in the field. This could be a consequence of slow initial stem growth rates and investment in large leaves that are susceptible to pests.

The foliar N concentration vs. SPAD reading calibration confirms that the SPAD meter can serve as a suitable non-destructive surrogate for leaf N. Weih and Rönnberg-Wastljüng (2007) demonstrated the usefulness of a SPAD meter in predicting canopy N in field grown shrub willow, where after excluding leaves with SPAD readings below 20, the authors reported an R^2 of 0.82 ($P < 0.001$) between foliar N concentration and SPAD. The relationship between SPAD and foliar N has also been reported in two species of poplar (*Populus* spp.), another genus in the willow family (Salicaeae). The variation in foliar N concentration predicted by SPAD

ranged from R^2 = 0.40 (Bonneville and Fyles, 2006) to R^2 = 0.42 (Loh et al., 2002). The variation explained in the current study was R^2 = 0.68. The weekly SPAD readings should therefore represent a reasonable, non-destructive approximation of foliar N dynamics during this experiment. The fact that there was little difference in SPAD for the first three weeks of measurements suggests that uptake of fertilizer N was low due to undeveloped root systems and the plants may have been relying to a large degree on the N stored in the cutting. This was a general trend observed by Brereton et al. (2013), who used an ^{15}N tracer study to observe translocation of N resources in a pot study over one growing season. The initial SPAD readings from each cultivar might approximate the initial cutting N content and/or the relative ability for those cultivars to mobilize N from the cutting to the leaves. The *S. purpurea* cultivar, 'Fish Creek', and the (*S. koriyanagi* × *S. purpurea*) × *S. miyabeana* cultivar, 'Geneva Giant', had very rapid increases in SPAD and tended to remain high throughout the experiment, suggesting a heavy N investment in leaves. Based on stem dry matter production and the stem/leaf biomass ratio this seemingly large investment in leaf canopy N did not translate into greater biomass for these two cultivars. Interestingly, the two *S. purpurea* × *S. miyabeana* cultivars began at a low level and remained rather low, far lower than the *S. purpurea* cultivar 'Fish Creek' and the *S. miyabeana* cultivar, 'SX61'. LA970253 and 'Fabius' had a high initial SPAD and maintained high readings through the first half of the experiment, followed by a rapid decrease in the second half. Perhaps this could indicate a relatively high level of cutting N or a high rate of translocation from the cutting to the leaves early in the growth period. These and the other two *S. viminalis* × *S. miyabeana* triploid cultivars had the greatest starting total stem length. Brereton et al. (2013) suggested that genotypes with greater final biomass production had a growth strategy of earlier utilization or depletion of cutting N, which may be the case for these triploids. The general

downward trend in SPAD reading towards the end of the experiment could represent a dilution effect with increasing biomass, or an inability of the supplied fertilization to keep up with plant demand. Remarkably, plants receiving the water-only control treatment were not significantly different from the F1 treatment group for a number of measured traits and mostly deficient in plant foliar production, but there was no significant difference in single-leaf SPAD reading at the end of the experiment.

The cultivars tested for response to fertilization in this controlled environment experiment represented a wide range of genetic diversity and included genotypes from major breeding programs. Due to conflicting results from field studies on nutrient response found in the literature, controlled environment fertilization trials may help to identify growth and physiological traits that are related to high biomass productivity. The range of fertilization treatments selected in this study were not extreme and were representative of those found in the literature for other shrub willow pot studies. There were few significant cultivar-by-treatment interactions and those that were significant were relatively weak compared the main effects and were likely due to occasional rank changes within fertilization levels. Despite the potential for generating misleading conclusions from short-term pot studies, final total dry aboveground biomass production among cultivars in this study were fairly well correlated with field trial yields with one or two exceptions. Based on leaf traits and growth modeling, strategies for greater total and stem biomass production seemed to be associated with either large investments in total foliar biomass or the efficiency of photosynthesis of those leaves. Future work involving the measurement of photosynthesis traits in response to fertilization of high-yielding cultivars would likely complement the findings of this study.

5.6 References

Andralojc PJ, Bencze S, Madgwick PJ, Philippe H, Powers SJ, Shield I, Karp A, Parry MAJ (2014) Photosynthesis and growth in diverse willow genotypes. Food Energy Secur. 3:69-85.

Aronsson P, Rosenqvist H, Dimitriou I (2014) Impact of nitrogen fertilization to short-rotation willow coppice plantations grown in Sweden on yield and economy. Bioenerg Res. 7:993-1001.

Bonneville MC, Fyles JW (2006) Assessing Variations in SPAD-502 Chlorophyll Meter Measurements and Their Relationships with Nutrient Content of Trembling Aspen Foliage. Commun. Soil Sci. Plant Anal. 37:525-539.

Bouman OT, Sylliboy J (2012) Biomass allocation and photosynthetic capacity of willow (Salix spp.) bio-energy varieties. Forstarchiv. 83:139-143.

Brereton NJB, Pitre FE, Shield I, Hanley SJ, Ray MJ, Murphy RJ, Karp A (2014) Insights into nitrogen allocation and recycling from nitrogen elemental analysis and ^{15}N isotope labelling in 14 genotypes of willow. Tree Physiol. 34:1252-1262.

Fabio ES, Kemanian AR, Montes F, Miller RO, Smart LB (2017a) A mixed model approach for evaluating yield improvements in interspecific hybrids of shrub willow, a dedicated bioenergy crop. Ind Crops Prod. 96:57-70.

Fabio ES, Volk TA, Miller RO, Serapiglia MJ, Gauch HG, Van Rees KCJ, Hangs RD, Amichev BY, Kuzovkina YA, Labrecque M, Johnson GA, Ewy RG, Kling GJ, Smart LB (2017b)

Genotype × environment interaction analysis of North American shrub willow yield trials confirms superior performance of triploid hybrids. GCB Bioenergy. 9:445-459.

Hangs RD, Schoenau JJ, Van Rees KCJ, Knight JD (2012) The effect of irrigation on nitrogen uptake and use efficiency of two willow (Salix spp.) biomass energy varieties. Can J Plant Sci. 92:563-575.

Hangs RD, Schoenau JJ, Van Rees KCJ, Steppuhn H (2011) Examining the salt tolerance of willow (Salix spp.) bioenergy species for use on salt-affected agricultural lands. Can J Plant Sci. 91:509-517.

Karp A, Hanley SJ, Trybush SO, Macalpine W, Pei M, Shield I (2011) Genetic improvement of willow for bioenergy and biofuels. J Integr Plant Biol. 53:151-165.

Karp A, Shield I (2008) Bioenergy from plants and the sustainable yield challenge. New Phytol. 179:15-32.

Kuzovkina YA, Quigley MF (2005) Willows beyond wetlands: Uses of Salix L. species for environmental projects. Water Air Soil Pollut. 162:183-204.

Kuzovkina YA, Weih M, Romero MA, Charles J, Hust S, McIvor I, Karp A, Trybush S, Labrecque M, Teodorescu TI, Singh NB, Smart LB, Volk TA (2008) Salix: Botany and global horticulture. In: Janick J (ed) Horticultural Reviews. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp 447-489.

Littell RC, Pendergast J, Natarajan R (2000) Modelling covariance structure in the analysis of repeated measures data. Stat Med. 19:1793-1819.

Littell RC, Stroup WW, Milliken GA, Wolfinger RD, Schabenberger O (2006) SAS for mixed models, Second Edit. SAS institute Inc., Cary, NC, USA.

Loh FCW, Grabosky JC, Bassuk NL (2002) Using the SPAD 502 meter to assess chlorophyll and nitrogen content of benjamin fig and cottonwood leaves. Horttechnology. 12:682-686.

Mamashita T, Larocque GR, DesRochers A, Beaulieu J, Thomas BR, Mosseler A, Major J, Sidders D (2015) Short-term growth and morphological responses to nitrogen availability and plant density in hybrid poplars and willows. Biomass Bioenerg. 81:88-97.

Meredith MP, Stehman SV (1991) Repeated measures experiments in forestry: focus on analysis of response curves. Can J For Res. 21:957-965.

Paine CET, Marthews TR, Vogt DR, Purves D, Rees M, Hector A, Turnbull LA (2012) How to fit nonlinear plant growth models and calculate growth rates: an update for ecologists. Methods Ecol Evol. 3:245-256.

Rönnberg-Wästljung AC, Gullberg U (1999) Genetics of breeding characters with possible effects on biomass production in Salix viminalis (L.). Theor Appl Genet. 98:531-540.

SAS Institute Inc. 2013. SAS Institute, Inc, Cary, NC, USA.

Serapiglia MJ, Gouker FE, Smart LB (2014) Early selection of novel triploid hybrids of shrub willow with improved biomass yield relative to diploids. BMC Plant Biol. 14:74.

Stoof CR, Richards BK, Woodbury PB, Fabio ES, Brumbach AR, Cherney J, Das S, Geohring L, Hansen J, Hornesky J, Mayton H, Mason C, Ruestow G, Smart LB, Volk TA, Steenhuis

TS (2015) Untapped potential: Opportunities and challenges for sustainable bioenergy production from marginal lands in the Northeast USA. Bioenerg Res. 8:482-501.

Taylor G, Allwright MR, Smith HK, Polle A, Wildhagen H, Hertzberg M, Bhalerao R, Keurentjes JJB, Scalabrin S, Scaglione D, Morgante M (2016) Bioenergy trees: Genetic and genomic strategies to improve yield. In: Barth S, Murphy-Bokern D, Kalinina O, Taylor G, Jones M (eds) Perennial biomass crops for a resource-constrained world. Springer International Publishing, Cham, pp 167-190.

Tharakan PJ, Volk TA, Nowak CA, Abrahamson LP (2005) Morphological traits of 30 willow clones and their relationship to biomass production. Can J For Res. 35:421-431.

U.S. Department of Energy (2016) 2016 Billion-Ton Report: Advancing Domestic Resources for a Thriving Bioeconomy, Volume 1: Economic Availability of Feedstocks. M. H. Langholtz, B. J. Stokes, and L. M. Eaton (Leads), ORNL/TM-2016/160. Oak Ridge National Laboratory, Oak Ridge, TN. 448p

Weih M (2001) Evidence for increased sensitivity to nutrient and water stress in a fast-growing hybrid willow compared with a natural willow clone. Tree Physiol. 21:1141-1148.

Weih M (2009) Genetic and environmental variation in spring and autumn phenology of biomass willows (Salix spp.): effects on shoot growth and nitrogen economy. Tree Physiol. 29:1479-1490.

Weih M, Nordh NE (2002) Characterising willows for biomass and phytoremediation: growth, nitrogen and water use of 14 willow clones under different irrigation and fertilisation regimes. Biomass Bioenerg. 23:397-413.

Weih M, Nordh NE (2005) Determinants of biomass production in hybrid willows and prediction of field performance from pot studies. Tree Physiol. 25:1197-1206.

Weih M, Ronnberg-Wastljung AC (2007) Shoot biomass growth is related to the vertical leaf nitrogen gradient in Salix canopies. Tree Physiol. 27:1551-1559.

CHAPTER 6

Overall conclusions and future directions

One main overall goal of this dissertation research was to examine yields and yield stability among commercial and pre-commercial cultivars in order to better understand the genotype-by-environment interactions in shrub willow during the first three-year rotation period (Chapters 2 and 3). Yield is arguably the most important factor affecting the profitability and sustainability of biomass feedstock production, but biomass composition can impact the end use and energy conversion efficiency of the feedstock. Therefore, it is also important to understand the genetic and environmental variation affecting biomass composition (Chapter 4). Finally, there has been much debate in the literature as to what the nutrient demands of SRC shrub willow are and a quantitative synthesis of the literature was used to demonstrate the range of response to fertilization (Chapter 1). This analysis showed in part that the response at the species or species hybrid level can be highly variable. This was confirmed in a controlled environment study conducted with newly bred cultivars (Chapter 5). Overall the work embodied in this dissertation serves to complement the breeding research that has been accomplished in North Amercia over the last three decades. Genetic improvement and agronomic research applied together can provide a synergy needed for successful crop development. Below is a summary of the important findings of this PhD research, along with knowledge gaps that are still present and some recommendations for future research.

The additive main effects and multiplicative interactions, or AMMI analysis, provides an elegant framework for cutting through the inherent noise of yield trial, or multi-environment trial datasets. An extensive body of work over several decades has sought to quantify the impact of

genotype-by-environment interactions with the aim of identifying stable or adaptable cultivars for numerous crops and the AMMI model is proving to be a favored method. To the best of my knowledge, this work was the first application of the AMMI model to shrub willow (Chapter 2). It provided a solid confirmation that triploid hybrids have superior yields over diploid and tetraploid cultivars. It also demonstrated that these triploids can display extremes in sensitivity and adaptability to environmental conditions, even amongst closely related individuals. The interaction scores resulting from the AMMI analysis were used to identify environmental factors that may be important for determining yields across broad geographic ranges. Growing degree days and precipitation were significantly correlated with overall yields, while minimum annual temperatures and growing degree days were correlated with interaction scores, suggesting differential reactions to temperature factors among cultivars. Edaphic factors were not found to be significantly correlated with overall yields or interactions, but the wide-ranging geographic variation in the dataset was likely greater than soil characteristics. Soil data obtained from publically available databases may be too course in scale and don't account for field history. For future yield trial network establishment, a unified soil sampling and testing protocol should be adopted and implemented at the start of each trial.

The main disadvantage of the fixed effects AMMI analysis is the requirement for a balanced dataset, which means that valuable information may need to be discarded if not all genotypes are present in all test environments. The implementation of mixed model interaction analyses can eliminate this limitation and the comparison of numerous mixed model versions of classical interaction analyses are beginning to show that the mixed model analog of AMMI often has the ability to explain the most variance in numerous datasets from diverse crops. This was the case for the most part with the shrub willow yield data and the use of the mixed model

AMMI confirmed previous findings, but with the ability to increase the available data incorporated in the analysis by 50%. The mixed model AMMI also helped to identify numerous newly bred cultivars that have robust performance and are thus appropriate for commercial scale-up. This work also provided a framework for analyzing future breeding material. Future efforts should include the analysis of multiple harvest rotations in order to truly assess yield stability.

Since biomass quality can also be an important factor affecting the end utilization of bioenergy crops, an extensive collection of biomass samples was analyzed in order to explore the genotypic, environmental and interaction contributions to variation in willow biomass composition. This analysis showed that ash content is under strong genetic control, which is relevant, since ash can negatively impact the efficiency of some energy conversion pathways. Since the economic viability of bioenergy production is directly reliant on the efficiencies of the internal processes, it might be beneficial to recommend certain cultivars over others for a particular conversion process. The composition analysis also revealed that optimal cellulose production on a per area basis can be achieved through selection of cultivars with high cellulose content, but also through the selection of cultivars based solely on yield, since both options produce the greatest amounts of cellulose. It has not been tested, however, whether both strategies would yield the same amounts of liquid fuels since other biomass components such as lignin have been shown to reduce the efficiency of liquid fuel production for some feedstocks.

Finally, the testing of elite cultivars for the response to fertilization should help to tailor the application of shrub willow to the needs of a particular project. For instance, if willow is being grown for biomass production on marginal land with low nutrient availability, then selecting cultivars with high nutrient use efficiencies would be beneficial. On the other hand, if shrub willow is being used in a situation such a riparian buffer strips to filter out nutrients in

runoff from adjacent agricultural fields, then a cultivar with luxury nutrient uptake might be best to maximize the filtering effect. However, pot studies are limited in the sense that it is difficult to extrapolate results to those that might be expected for plants grown in field situations. Therefore, similar testing of physiological responses to fertilization in field trials should be implemented. There were large genetic differences in the species and species hybrids tested in this research, and so perhaps future research could use numerous individuals from multiple families to test for variation in response among related individuals. This would be helpful for selecting individuals for future crosses and may also allow for finding genetic markers that are associated with fertilizer response.

Overall, this research demonstrated the usefulness of applied research in crop development. We are far from being able to formulate nutrient recommends to accompany soil nutrient analyses, like those that have been developed for so many field and vegetable crops. However, this research has helped to identify knowledge gaps that can hopefully be filled in order to eventually advance shrub willow production in a future with uncertain climatic and energy conditions.

APPENDIX 1

Implementation of cover crops for improved shrub willow establishment through weed and nutrient management

Background and justification

The establishment phase of shrub willow, which encompasses the first two years of production, represents nearly one quarter of overall cost to production and poor establishment can lead to increased management costs and could potentially reduce yield over the life of the crop. Weed competition in the first two years is generally considered the greatest challenge to crop success, since weed pressure can severely impact survival and growth if not properly managed. Current recommendations for weed management rely heavily on the use of a combination of pre- and post-emergence herbicides to maintain low levels of weed establishment through the first two years of willow growth to minimize weed competition until the willow crop can produce enough above ground biomass to close the canopy. Repeated applications of herbicides are expensive, can lead to an increased high risk of soil erosion, degradation of soil health, contribute to environmental contamination and can promote herbicide resistant weed populations. Incorporating cover crops into shrub willow cropping systems should be a way to achieve weed management goals for the successful establishment of the crop, and reduce the need for herbicides, which would lead to more sustainable and economical production practices. However, competition between cover crops and willow should be minimized. Integrating reduced tillage practices, such as zone building, with cover crop management should help to reduce interspecific competition and reduce the potential for soil erosion.

Controlling weed competition is a critical component of shrub willow cultivation during establishment due to the crop's poor competitive ability (Mitchell et al. 1999). Reductions in first

year growth of between 50 and 95% have been reported when weeds are left unchecked, which can threaten the profitability of the operation (Sage 1999). Recommendations in the Northeast formed nearly 15 years ago for weed control in the establishment of willow crops stress the need for mechanical and chemical treatment (Abrahamson et al. 1998), but little progress has occurred in improving the sustainability of these practices. These recommendations create a significant potential for soil erosion to occur in the first two years of establishment because intensive weed management results in exposure of mineral soil during most of the establishment period (Volk et al. 2004).

Cover crops have been encouraged in willow cropping systems in the Northeast, where winter cereal crops are seeded in the fall and terminated the following spring by incorporation into the soil with conventional tillage prior to planting willow (Volk et al. 2006). This may help to retain soil and nutrients on site over the winter and suppress winter annual weeds, but there would be limited suppression capability after willow is planted, which usually occurs after the danger of frost has passed. Intercropping cover crops with biomass energy crops shows great promise for controlling soil erosion during establishment (Malik et al. 2000), however, competition is certain to occur and will likely lead to yield losses (Malik et al. 2001). Adiele and Volk (2011) established Dutch white clover (*Trifolium repens*) in the spring prior to planting willow and reported excellent weed suppression, but first year willow yield was low and no different than an unweeded control, suggesting that intense competition occurred between willow and clover. They also included a glyphosate plus pre-emergence herbicide treatment that was applied in strips where willow was to be planted, which improved willow growth and reduced the amount herbicide applied relative to conventional practice. It is unclear, however, how this would translate to large, commercial scale production, since this practice would require

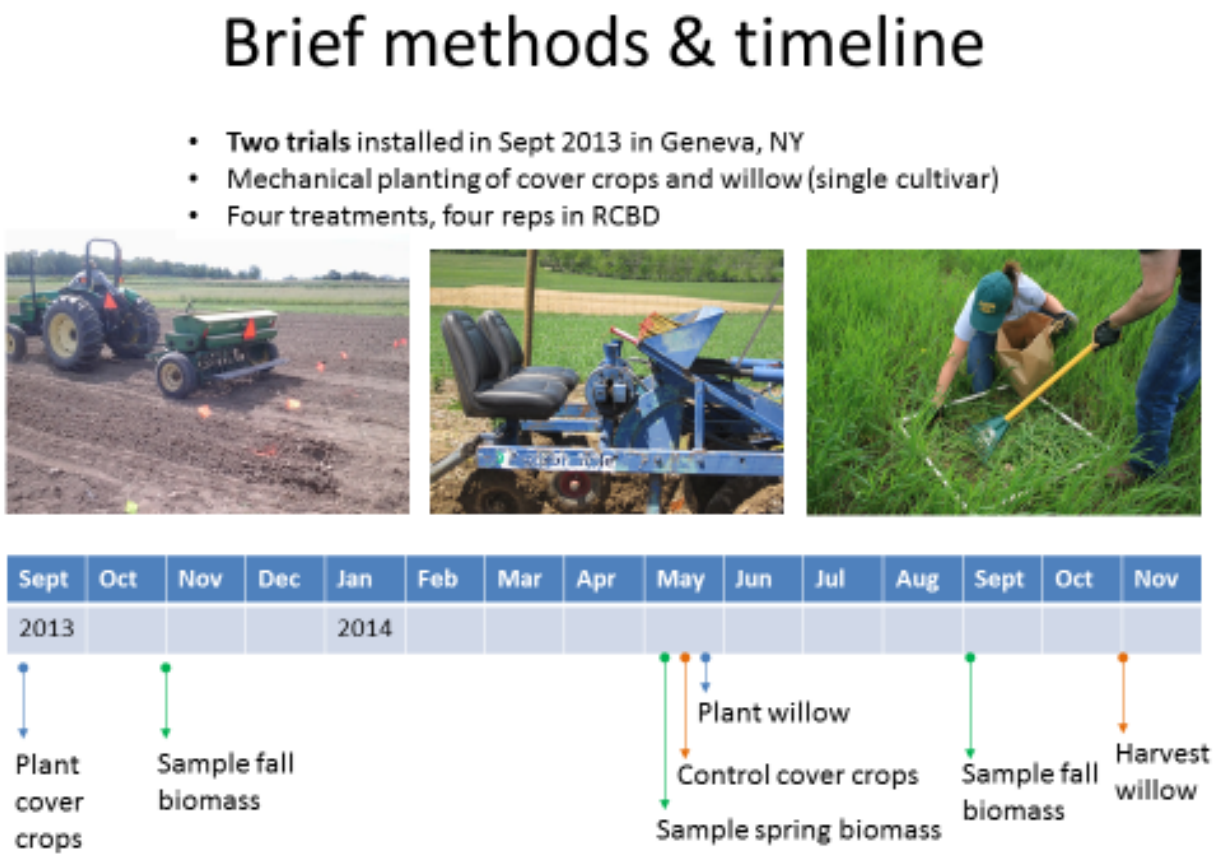
specialized sprayers and GPS-guided equipment. Similar results may be achievable by implementing GPS-guided zone tillage to create planting strips through an established cover crop, thus lessening the amount of exposed soil, while leaving a cover crop undisturbed between the rows of willows. Soil health may be improved while providing some weed suppression in the early stages of willow establishment. Cover crop termination near the time of willow planting is another means of reducing competition during the willow establishment phase, and if the cover crop is sown in the preceding fall, sufficient biomass may be produced to provide weed suppression during the early stages of willow growth and help to retain soil moisture (Teasdale and Mohler 1993). The decision to terminate the cover crop can be made after willow break bud, before completion occurs.

Nutrient additions are not normally applied in the first year of willow establishment out of concerns that the newly planted willow will not have enough biomass to assimilate the nutrients, and weed populations would benefit (Abrahamson et al. 1998). However, if banded nutrient applications were made at or near the time of planting, in combination with zone tillage where a weed-free strip would be created, nutrients would be concentrated near the newly expanding willow root systems. Banded fertilizer applications have been shown to be successful at increasing growth in hybrid poplar (Brown and van den Driessche 2005), but to our knowledge no published work using banded fertilizer applications in shrub willow production exists. This practice has potential to improve willow establishment and rooting, especially on phosphorus-deficient fields.

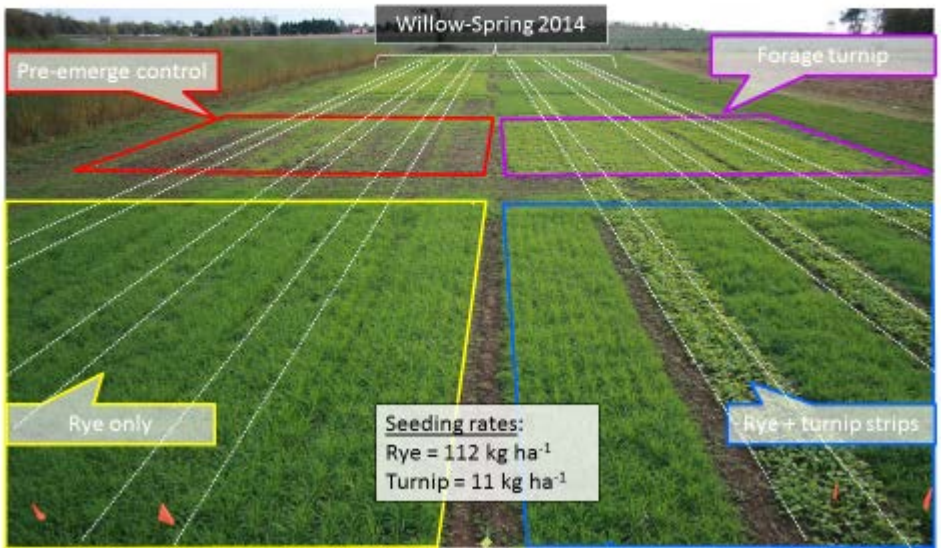
A series of experiments were implemented at Cornell's New York State Agricultural Experiment Station that aimed to incorporate cover crops and targeted nutrient applications to improve the sustainability of shrub willow establishment. The followed is a largely graphical

description of the procedures and outcomes of these experiments.

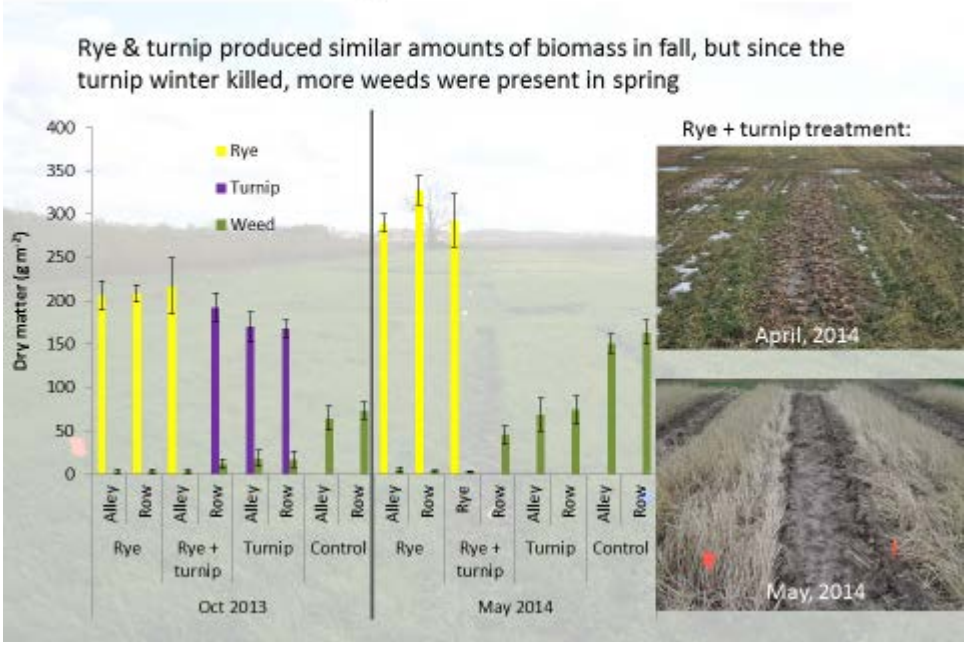
Trials 1 and 2: Testing cover crops and termination methods

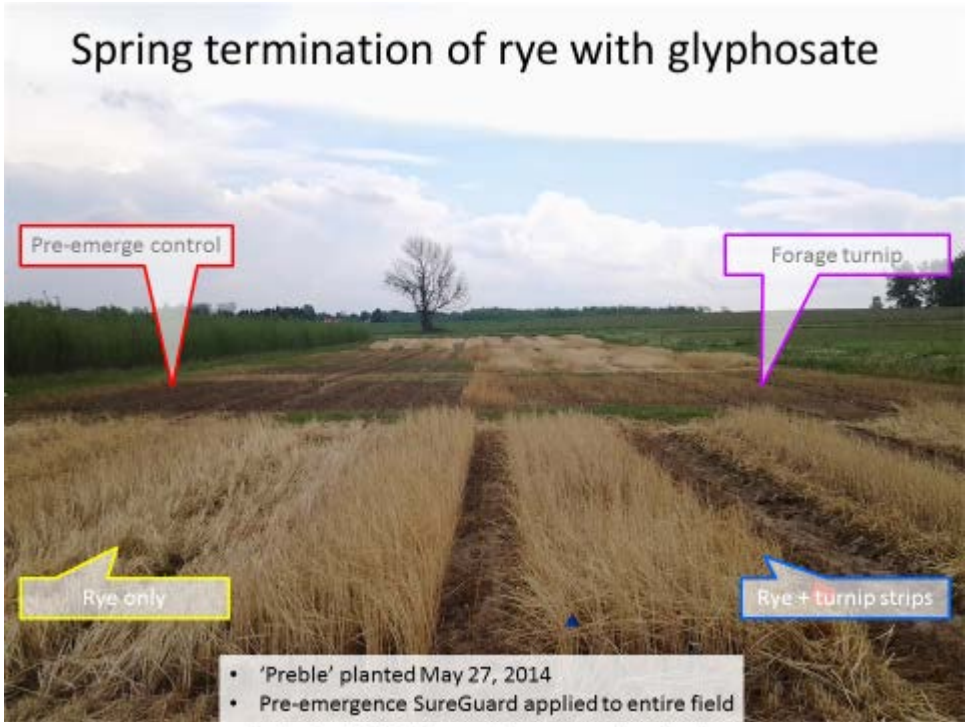


Trial 1: Fall seeded rye and brassica cover crops

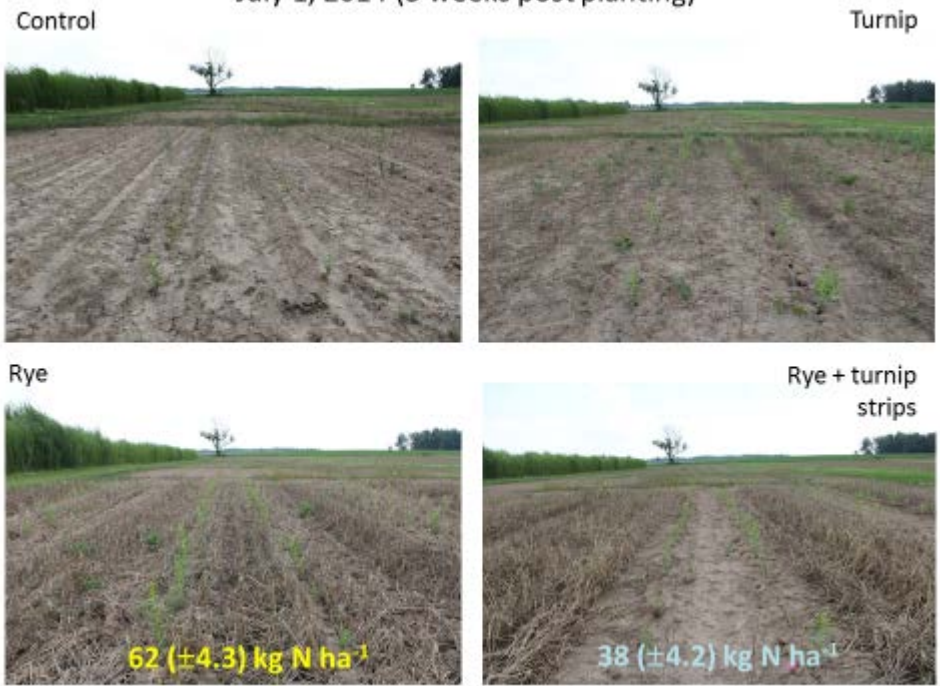


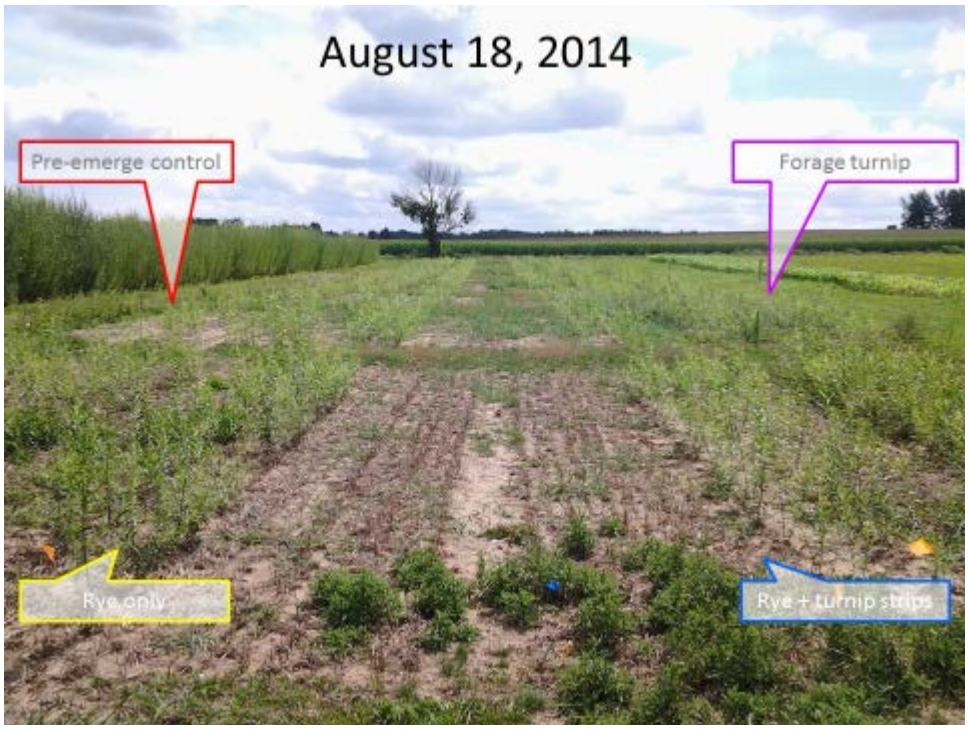
Cover crop and weed biomass

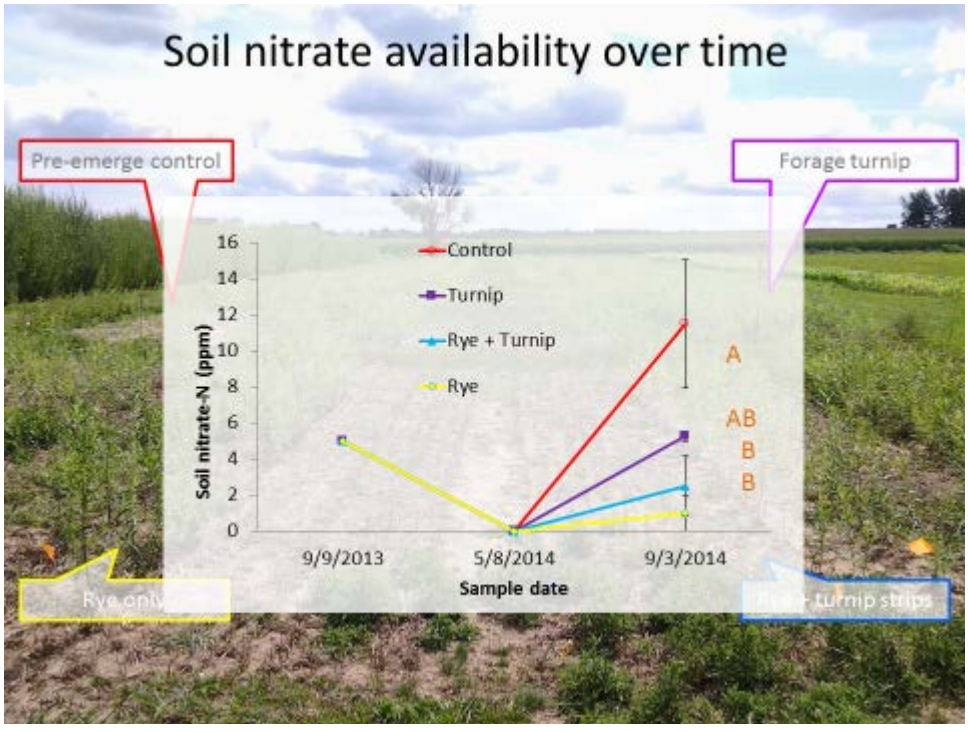


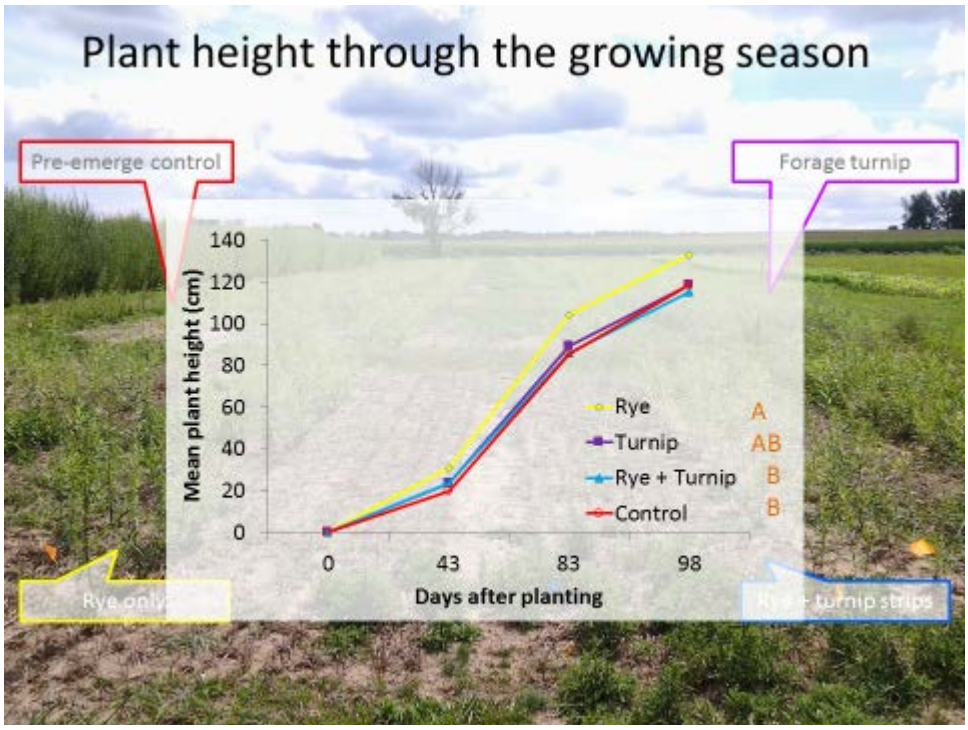


July 1, 2014 (5 weeks post planting)

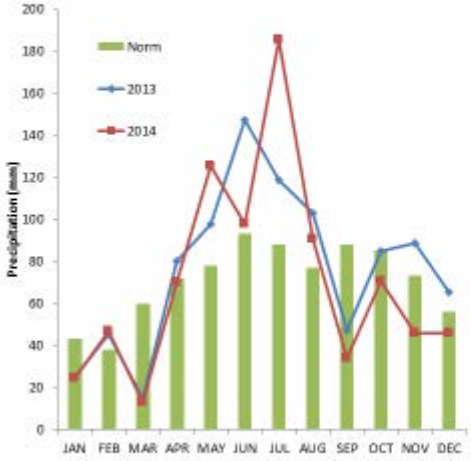


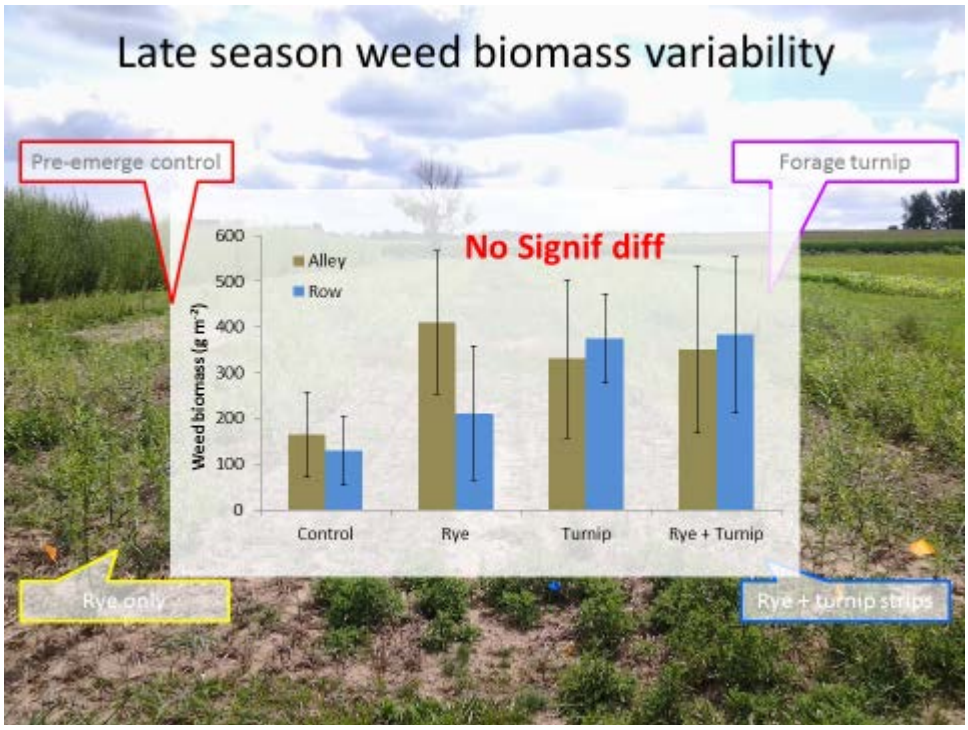

August 18, 2014
Pre-emerge control
Forage turnip
Rye only
Rye + turnip strips

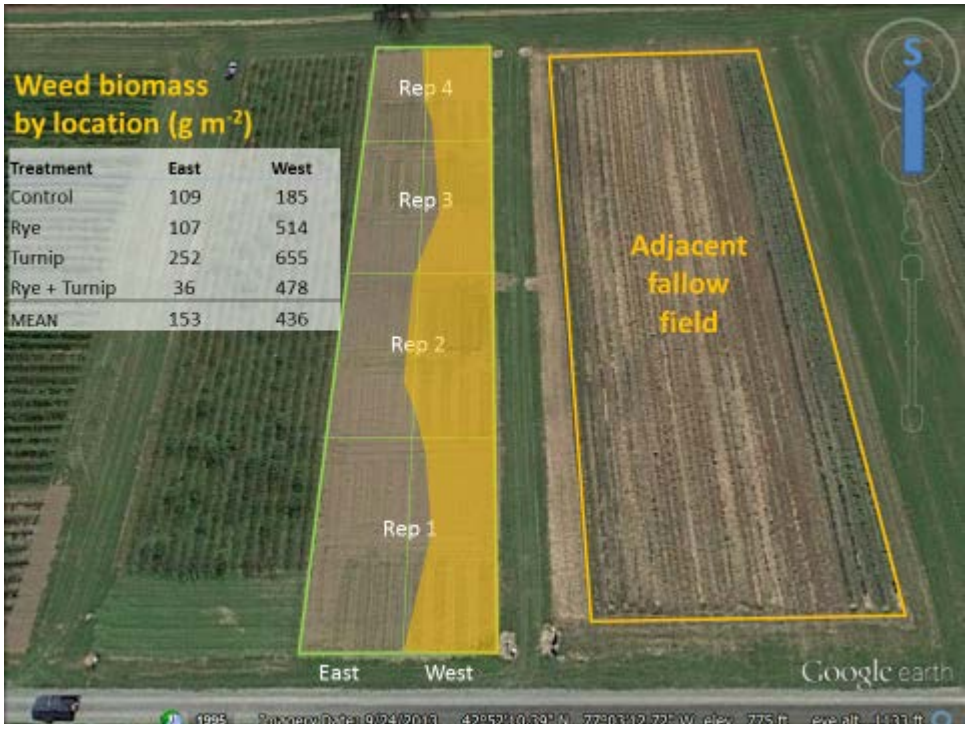

Soil nitrate availability over time
Pre-emerge control
Forage turnip
Control
Turnip
Rye + Turnip
Rye
Soil nitrate-N (ppm)
16
14
12
10
8
6
4
2
0
9/9/2013
5/8/2014
9/3/2014
Sample date
A
AB
B
B
Rye only
Rye + turnip strips

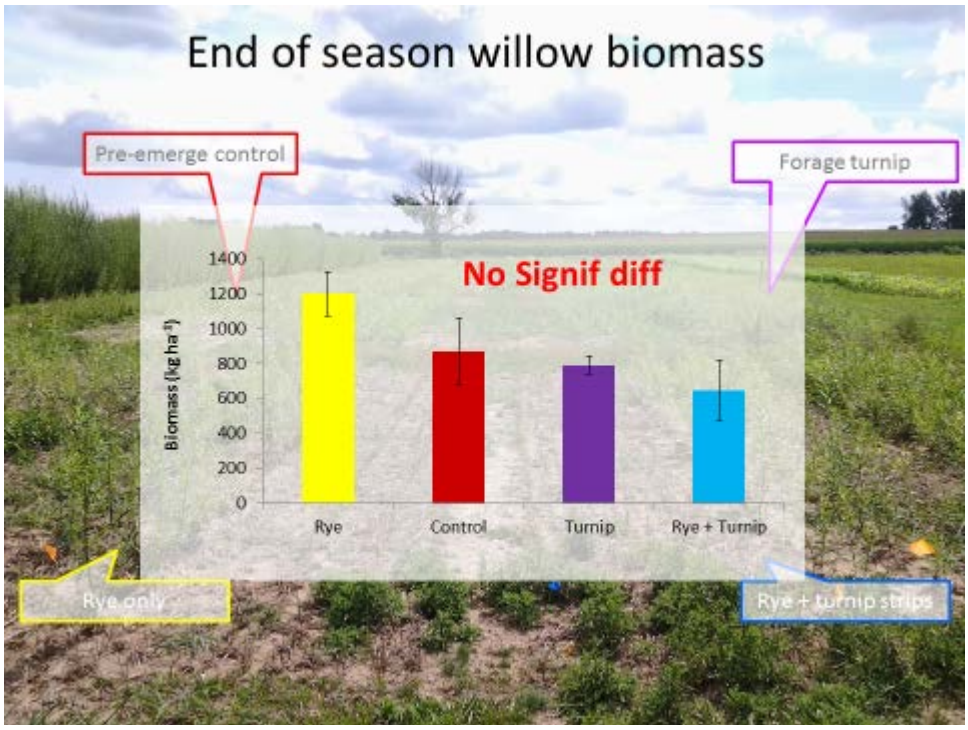


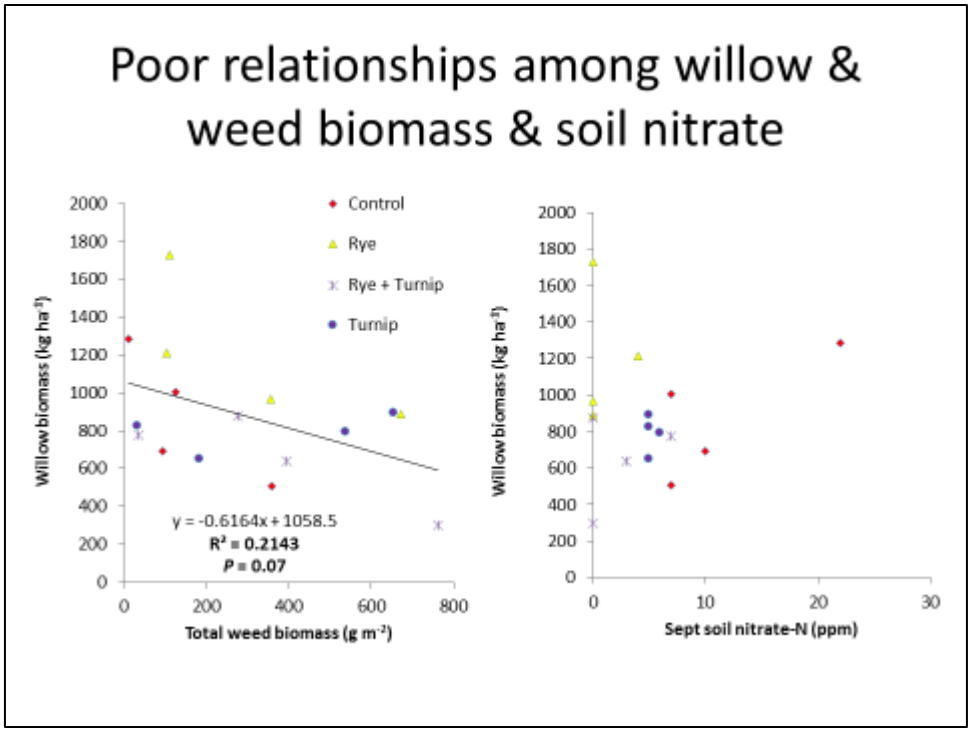
Possible causes for differences in growth




Late season weed biomass variability
Pre-emerge control
Forage turnip
No Signif diff
Alley
Row
Weed biomass (g m-2)
600
500
400
300
200
100
0
Control
Rye
Turnip
Rye + Turnip
Rye only
Rye + turnip strips

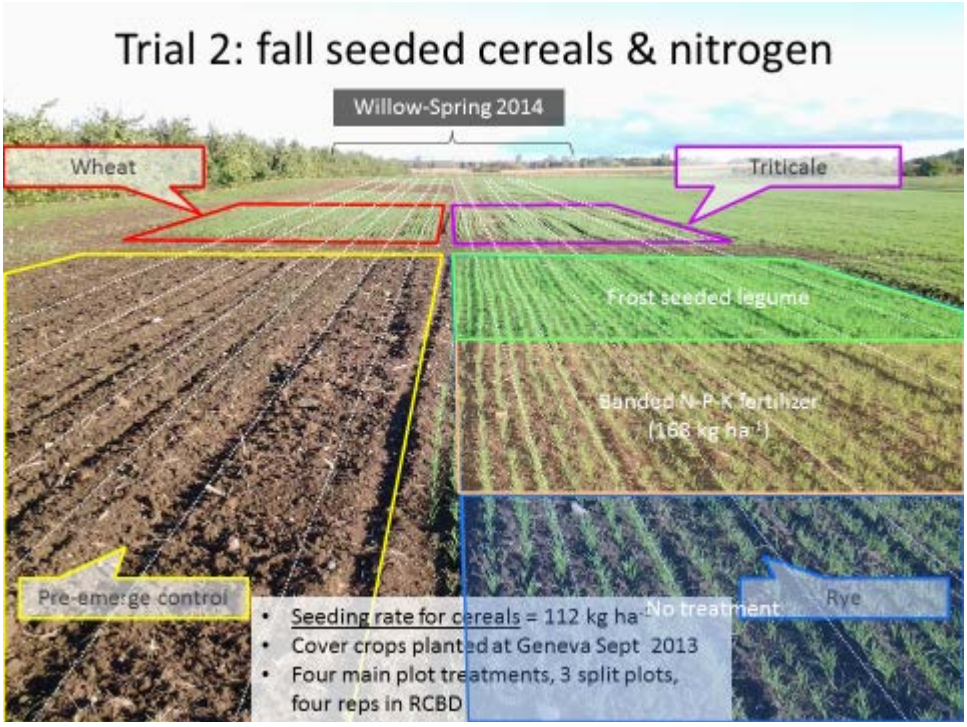

Weed biomass by location (g m-2)
Treatment East West
Control 109 185
Rye 107 514
Turnip 252 655
Rye + Turnip 36 478
MEAN 153 436
Rep 4
Rep 3
Rep 2
Rep 1
Adjacent fallow field
S
East
West
Google earth


End of season willow biomass
Pre-emerge control
Forage turnip
No Signif diff
Biomass (kg ha-1)
1400
1200
1000
800
600
400
200
0
Rye
Control
Turnip
Rye + Turnip
Rye only
Rye + turnip strips

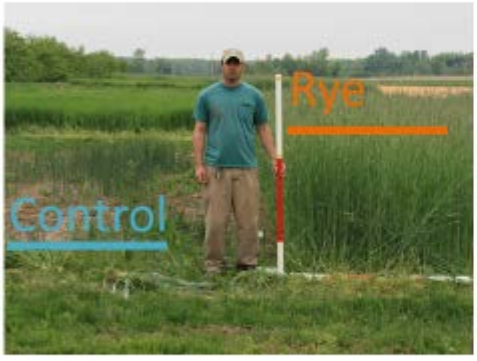

Poor relationships among willow & weed biomass & soil nitrate
Willow biomass (kg ha-1)
Control
Rye
Rye + Turnip
Turnip
y = -0.6164x + 1058.5
R² = 0.2143
P = 0.07
Total weed biomass (g m-2)
Sept soil nitrate-N (ppm)

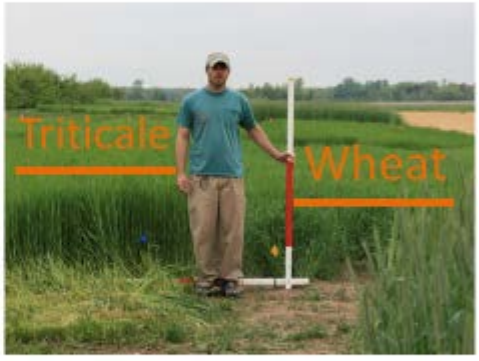
Trial 1 summary & conclusions

- Introducing cover crops into conventional site prep techniques had no adverse effects on willow growth
- Cover crop treatments reduced N availability, but did not affect willow growth
- Weed biomass spatial patterns influenced by environment
- Did willow benefit from cover ecosystem services?

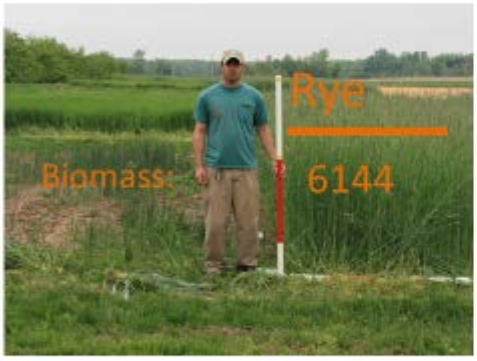


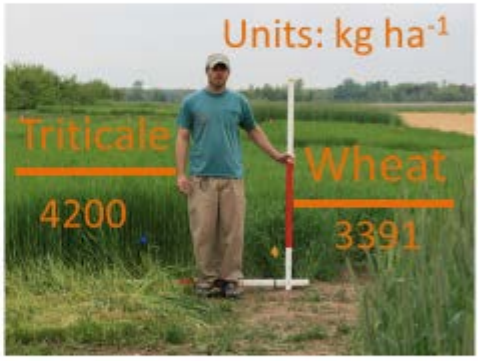
Cover crop growth by late May 2014



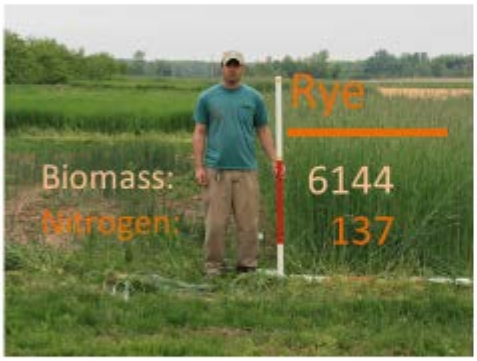


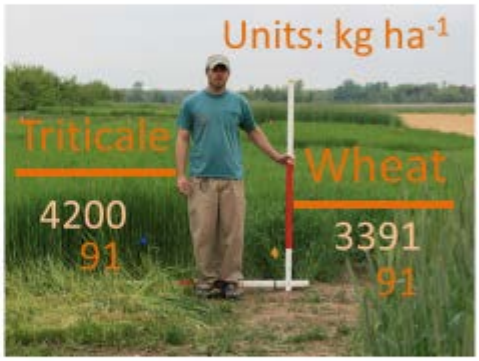
Biomass & N accumulation in spring





Biomass & N accumulation in spring





Cover crop management challenges

- Cover crops rolled on May 23, 2014 with a *Buffalo Rolling Stalk Chopper*
- Relatively good effect on rye
- Poor results in triticale and wheat due to later maturity

- Rolled 2nd time on June 2
- First rolling stimulated tillering in triticale and wheat

Cover crop management challenges

- Cover crops rolled on May 23, 2014 with a *Buffalo Rolling Stalk Chopper*
- Relatively good effect on rye
- Poor results in triticale and wheat due to later maturity

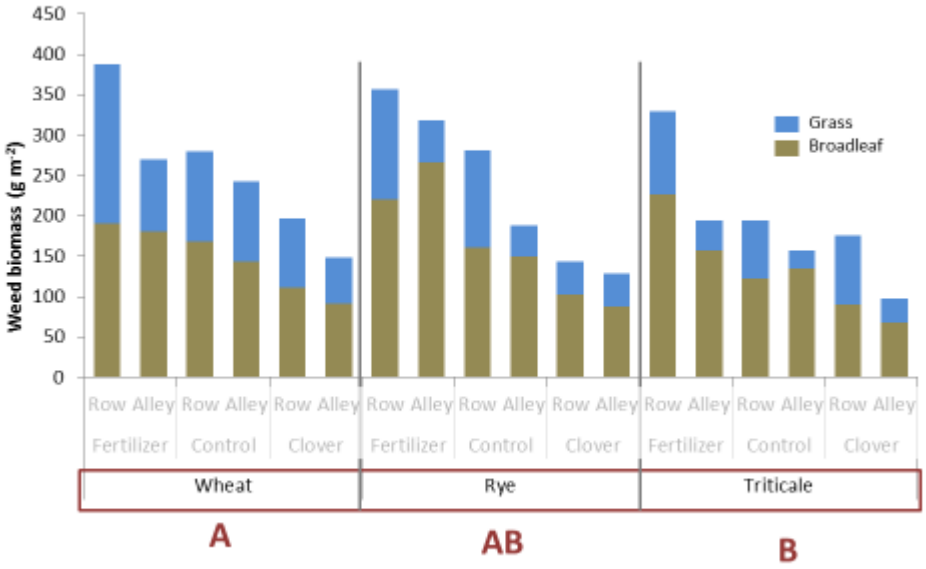
- Rolled 2nd time on June 2
- First rolling stimulated tillering in triticale and wheat

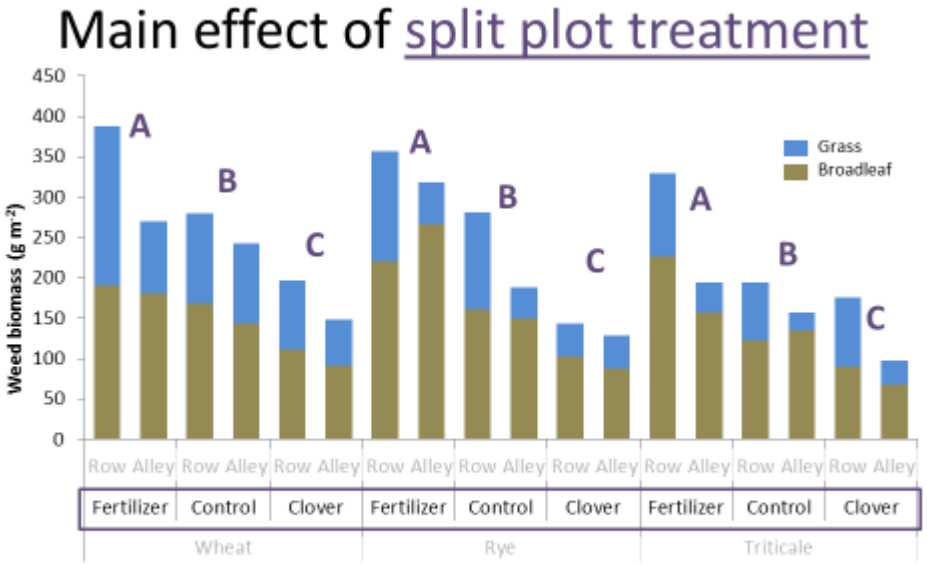
Cover crop management challenges

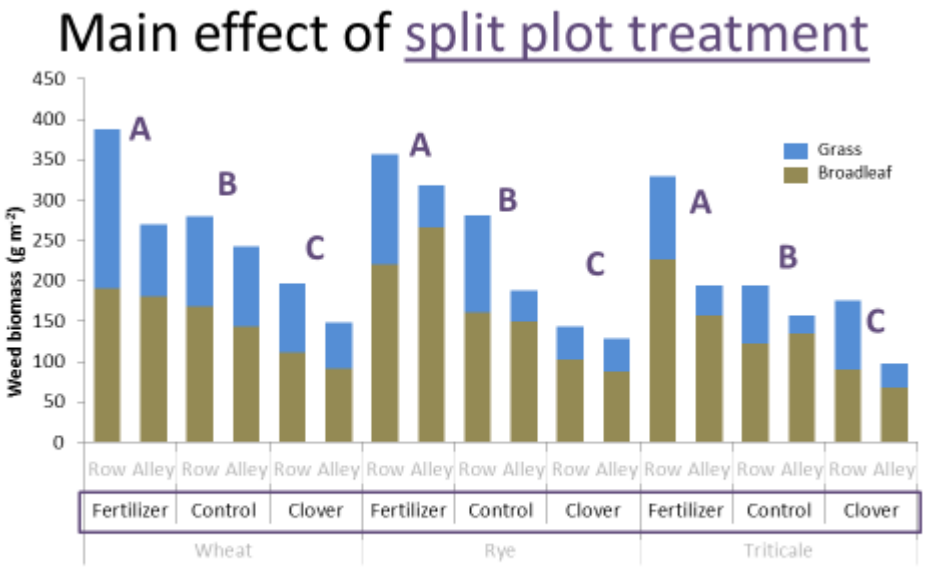
- Uneven ground reduces contact between blades and soil/cover crop
- Parallel linkage on each roller would improve contact (Steve Groff, PA)

- Zone tillage and mowing treatment likely contributed to the regular patterns in kill/no kill

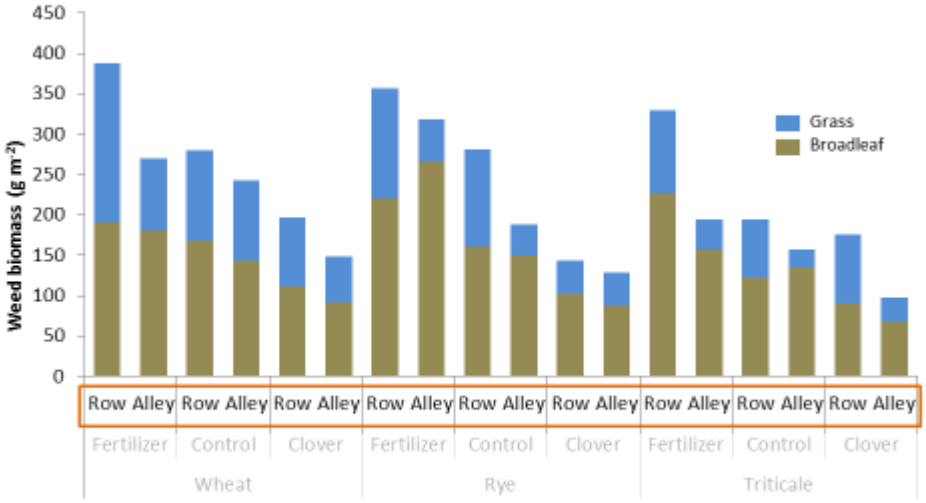
Main effect of cover crop species




Main effect of split plot treatment
Weed biomass (g m-2)
450
400
350
300
250
200
150
100
50
0
A
B
C
A
B
C
A
B
C
Grass
Broadleaf
Row Alley
Fertilizer
Control
Clover
Wheat
Rye
Triticale

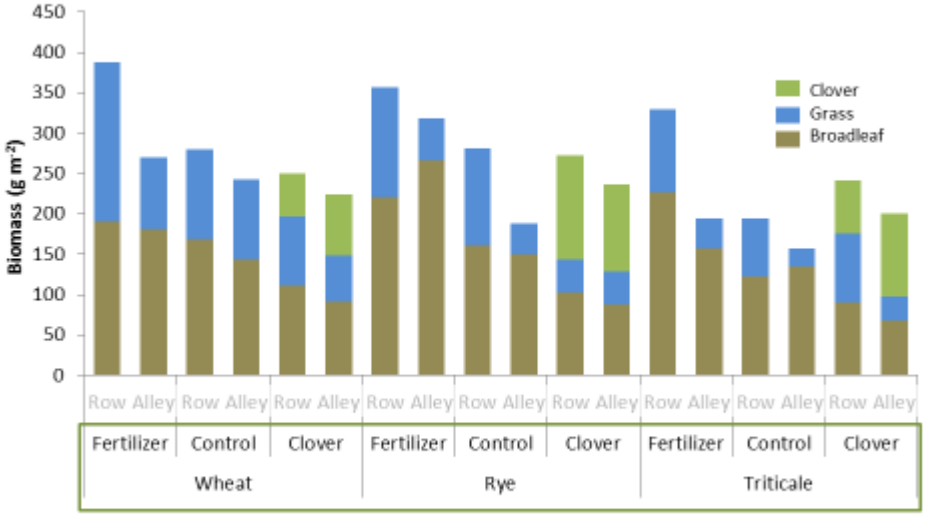

Main effect of split plot treatment
Weed biomass (g m-2)
450
400
350
300
250
200
150
100
50
0
A
B
C
A
B
C
A
B
C
Grass
Broadleaf
Row Alley
Fertilizer
Control
Clover
Wheat
Rye
Triticale

Main effect of position

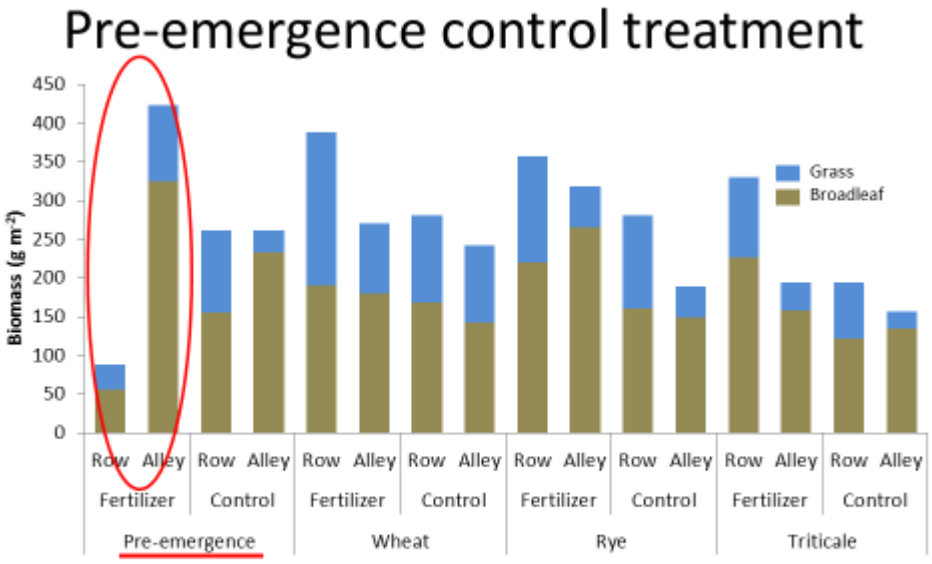


Row > Alley

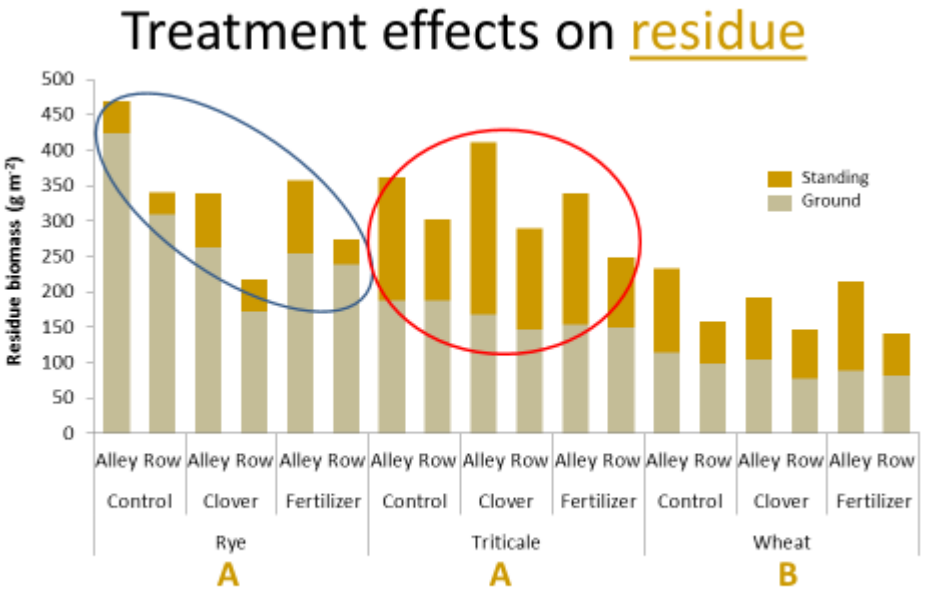
Total ground cover biomass



Wheat = Rye > Triticale **Fert > Control = Clover**

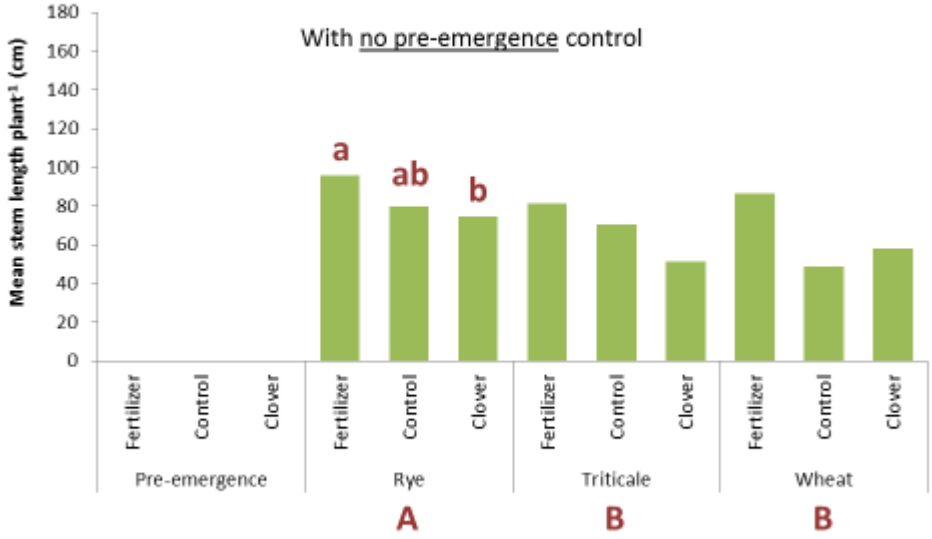


Signif Split Plot x Position **Fert > Control**



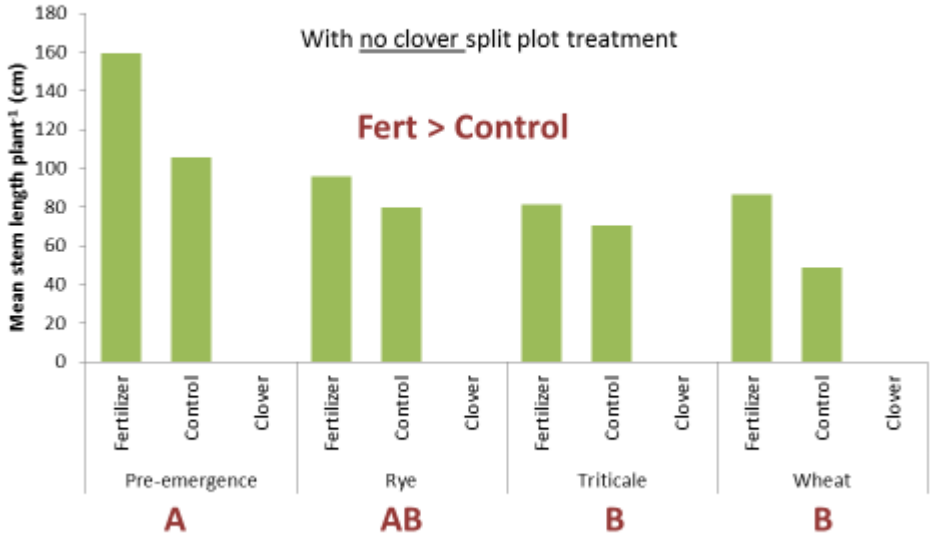
Control > Clover = Fert (p = 0.09) **Row > Alley**

What about the willow?



Alpha level $p < 0.10$

What about the willow?



Alpha level $p < 0.05$

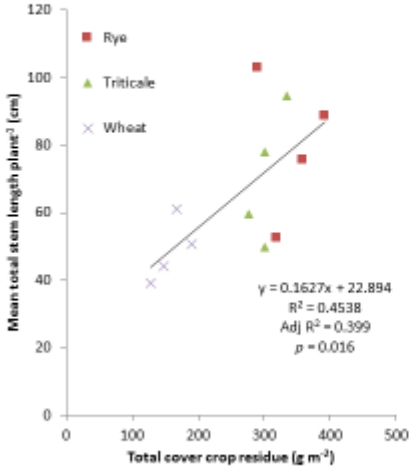
Some key observations in weed populations

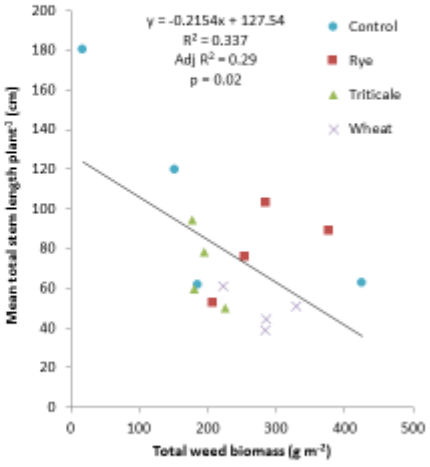
Competition between clover & willow

Weed biomass patchiness in controls

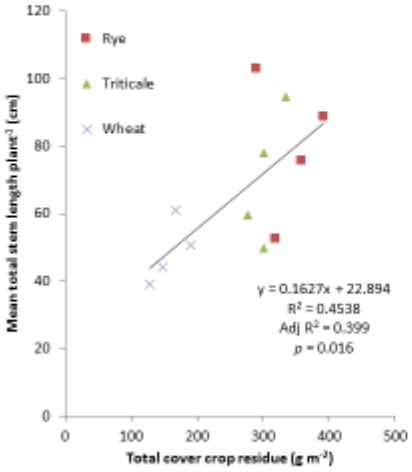
Tillering reduced weed biomass, but also willow

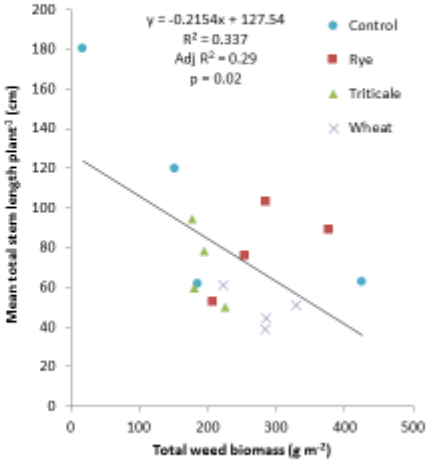
What factors are limiting willow growth?



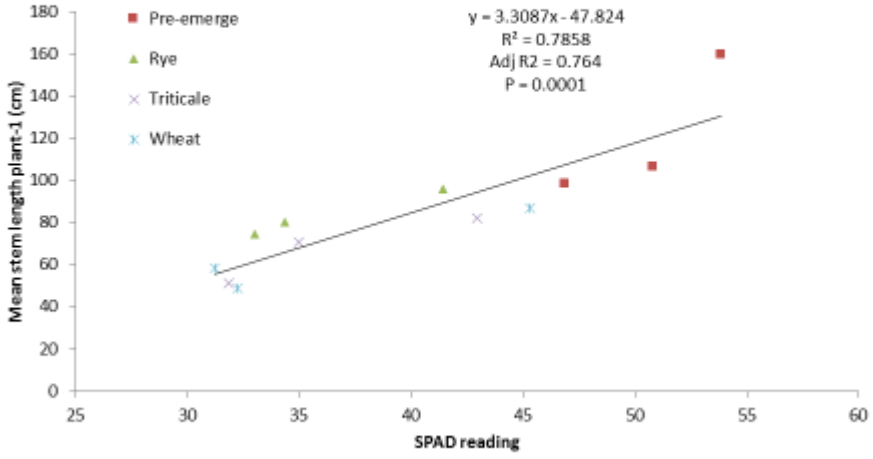


What factors are limiting willow growth?

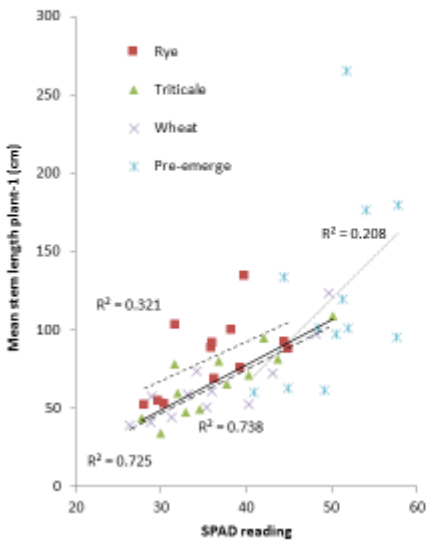


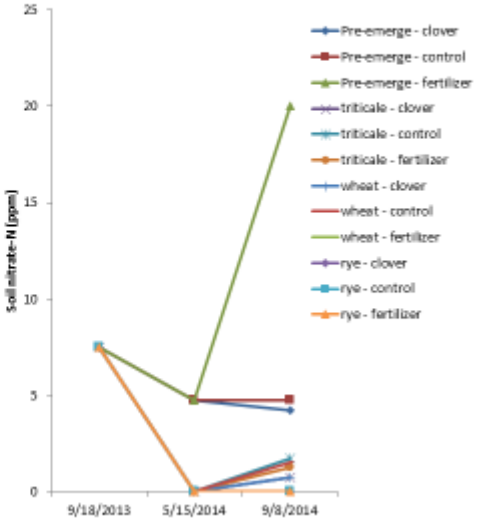


Is nitrogen status limiting growth?

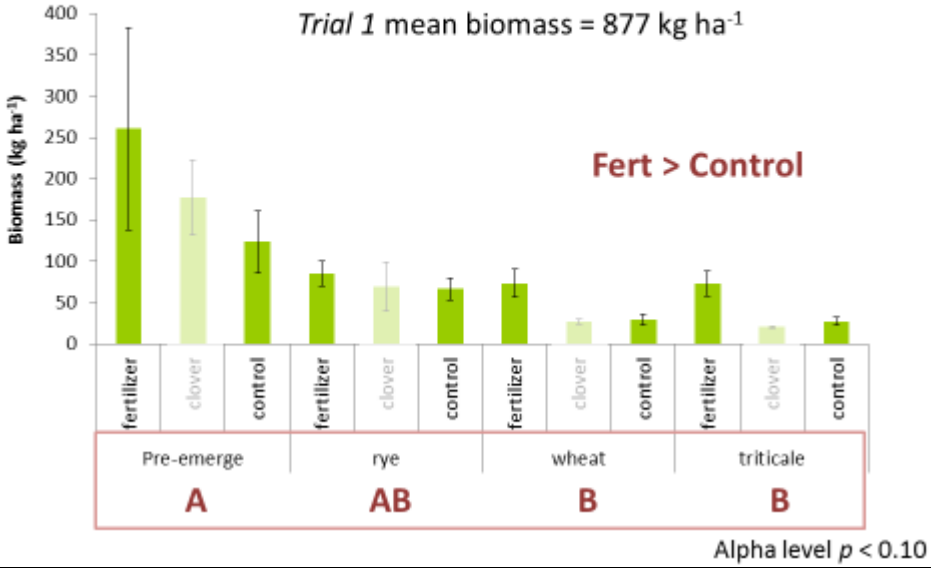


Is nitrogen status limiting growth?





End of season willow biomass yield



Alpha level $p < 0.10$

Trial 2 summary

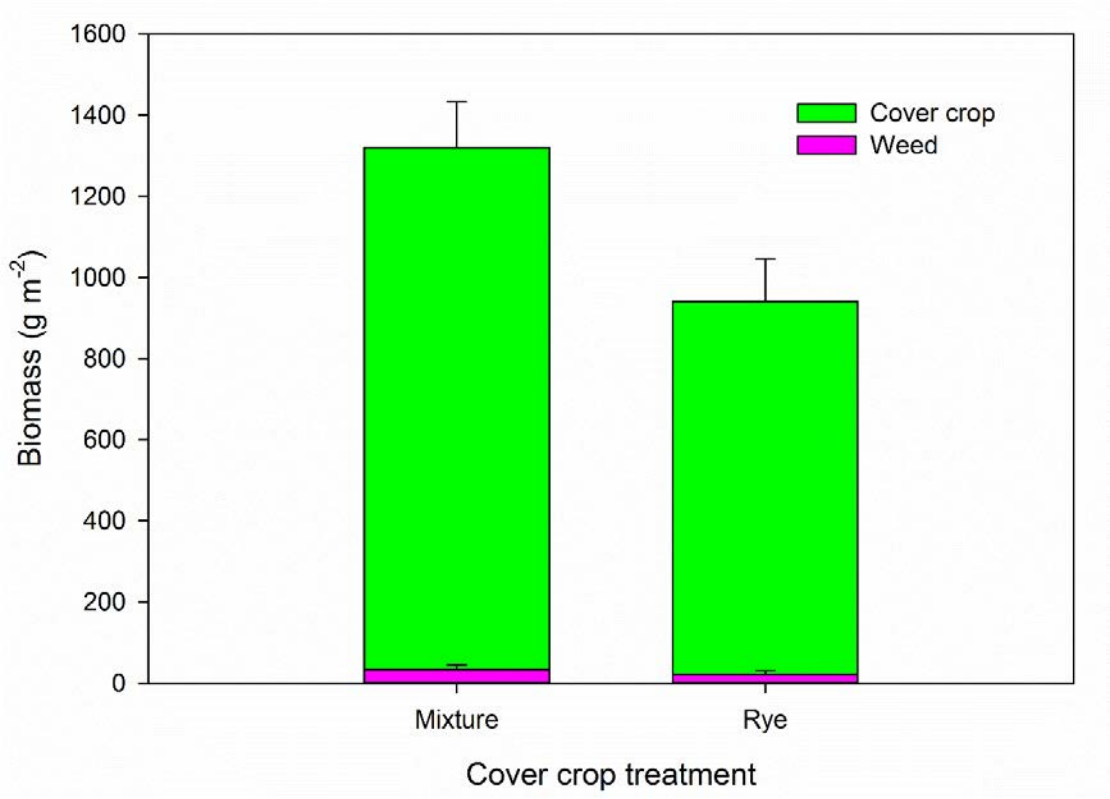
- Cereal cover crop species differed greatly in biomass & nitrogen accumulation
- Control equipment and timing of control are critical
- Increased amounts of residue seemed to benefit willow growth
- Increased nitrogen availability positively influenced both weeds & willow

Trial 3 sought to use fall-seeded cover crops in conjunction with the implement of precision agriculture technology for cover crop and nutrient management

- Three cover treatments in Fall 2015:
  - Fall-seeded cereal rye
  - Fall-seeded triticale, crimson clover and tillage radish mix
  - Fall plowing, fallow over winter
- GPS guidance was used to apply glyphosate in mid-May, 2016 in strips to cover crop treatment plots, and fully over control, fall-plowed plots
- A split-plot mowing treatment was also implementing after cover crop strip spraying

Fall plowed control plots on left after spring glyphosate application, next to the cover crop mixture treatment with crimson clover and triticale. The tillage radish had winterkilled. The glyphosate strip spray treatment can be seen at the far right.

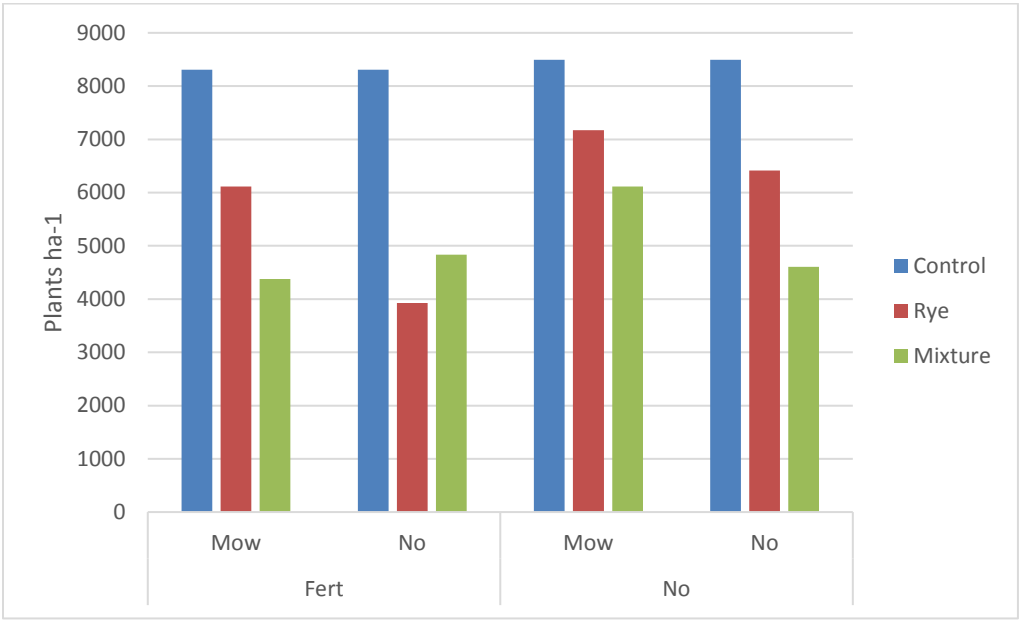
A split-split plot mowing treatment was applied prior to zone building for willow rows (see below).



By spring, 2016, cover crops were successful at suppressing weed biomass, but the mixture produced significantly more cover crop biomass than the rye only treatment. Weed biomass was not assessed in the control treatment, because the entire plots were sprayed with glyphosate in the spring, as would likely be done in a conventional setting.

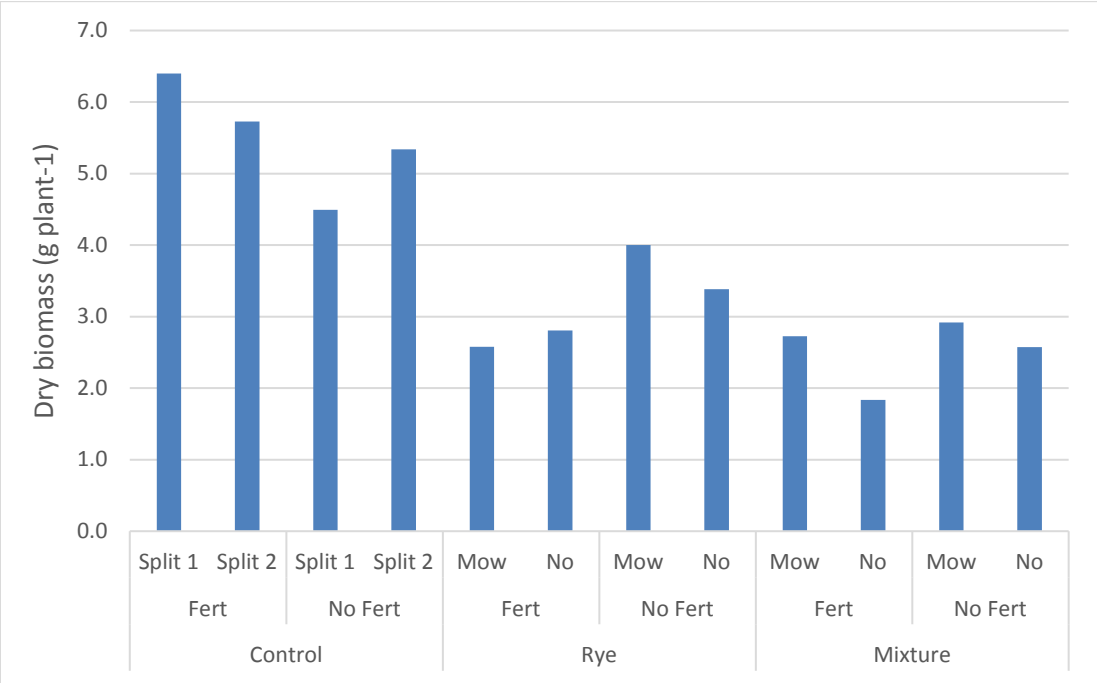
Zone tillage was implemented to establish willow rows using GPS guidance to align with previous strip spraying of glyphosate for cover crop control. A liquid fertilizer tank mounted to the zone builder was attached to pumps that fed drip lines behind each shank, delivering liquid 10-10-10 fertilizer at a rate of 27 kg N ha^{-1}. Cover crops growing between future willow rows were allowed to grow for weed control.

Willow planting was performed using a Egedal Energy Planter and the GPS coordinates used to establish the willow rows via zone builder were used during planting. There were severe problems with cover crop residue getting caught in the packing wheels of the planter, however, causing poor establishment of willow cuttings.



Overall, plant population establishment was poor, which was exacerbated by summer-long drought conditions, as the target density is approximately 15,000 plants ha^{-1}. However, the cover

crop treatments negatively impacted plant density and the greater biomass achieved by the mixture further reduced plant density estimates in most cases. Plant densities were slightly lower in the fertilizer treatments, likely due to increased weed pressure. Mowing had a positive impact on plant densities.



By August 2016, willow plant biomass followed a similar trend for the main effect of cover treatment, where the control had the greatest per plant biomass, followed by rye and then the mixture. The fertilizer treatment increased plant biomass in the control treatment, but suppressed it in the cover crop treatments, likely due to a fertilization effect on the weeds. In general mowing tended to increase plant biomass in the cover crop treatments. (Note: no mowing treatment was applied to the control plots, so data presented above for control split 1 and split 2 represent subsampling across those plots.)

General conclusions

- Overall, I think that cover crops definitely have a place in shrub willow establishment. At the very least they should be considered in conjunction with conventional herbicide regimes.
- If one goal is to reduce herbicide usage, then we should look at other species that winterkill, or using cover crops in combination with targeted applications of herbicides
- I am very interested in tested the roller-crimper that has been used extensively at Penn State, but the stalk chopper used here in Experiment 2
- But in the end, the added cost of planting and managing cover crops need to be weighed against willow outcomes and the benefits to ecosystem services.
- (~$74 ha^{-1} to plant rye, but what are the ecosystem services benefits, and what is their

value?)

- I don’t see feel there is a significant issue with nitrogen tie up and willow, at least not initially, so I think maximizing a mulch layer for weed suppression is something that still needs to be examined.
- One aspect to look at in the future is to determine the tolerable level of weed competition and what is the most sensitive timeframe for competition to occur.

References

Abrahamson, L. P., D. J. Robison, T. A. Volk, E. H. White, E. F. Neuhauser, W. H. Benjamin, and J. M. Peterson. 1998. Sustainability and environmental issues associated with willow bioenergy development in New York (U.S.A.). Biomass and Bioenergy 15:17-22.

Adiele, J. and T. A. Volk. 2011. Developing spring cover crop systems for willow biomass crop establishment. Aspects of Applied Biology 112:113–119.

Brown, K. R. and R. van den Driessche. 2005. Effects of nitrogen and phosphorus fertilization on the growth and nutrition of hybrid poplars on Vancouver Island. New Forests 29:89-104.

Malik, R. K., T. H. Green, G. F. Brown, C. A. Beyl, K. R. Sistani, and D. A. Mays. 2001. Biomass production of short-rotation bioenergy hardwood plantations affected by cover crops. Biomass and Bioenergy 21:21-33.

Malik, R. K., T. H. Green, G. F. Brown, and D. Mays. 2000. Use of cover crops in short rotation hardwood plantations to control erosion. Biomass and Bioenergy 18:479-487.

Mitchell, C. P., E. A. Stevens, and M. P. Watters. 1999. Short-rotation forestry – operations, productivity and costs based on experience gained in the UK. Forest Ecology and Management 121:123-136.

Sage, R. B. 1999. Weed competition in willow coppice crops: the cause and extent of yield losses. Weed Research 39:399-411.

Teasdale, J. R. and C. L. Mohler. 1993. Light transmittance, soil-temperature, and soil-moisture under residue of hairy vetch and rye. Agronomy Journal 85:673-680.

Volk, T. A., L. P. Abrahamson, C. A. Nowak, L. B. Smart, P. J. Tharakan, and E. H. White. 2006. The development of short-rotation willow in the northeastern United States for bioenergy and bioproducts, agroforestry and phytoremediation. Biomass and Bioenergy 30:715-727.

Volk, T. A., T. Verwijst, P. J. Tharakan, L. P. Abrahamson, and E. H. White. 2004. Growing fuel: a sustainability assessment of willow biomass crops. Frontiers in Ecology and the Environment 2:411-418.